THE USE OF RECOMBINANT CYTOKINES AS A NOVEL THERAPY TO IMPROVE HEALTH AND PRODUCTION IN DAIRY COWS

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ABSTRACT

During the transition from late gestation to early lactation, homeorhetic adaptations allow dairy cows to produce copious amounts of milk without compromising their own health. This transition, however, remains a potentially perilous time for dairy cows as it is associated with a high risk of infectious and metabolic disorders. These conditions have a negative impact on the dairy industry because they contribute to economic losses and compromise animal welfare. Despite decades of research dedicated to advancing knowledge and aiding in the prevention of periparturient disorders, their incidences remain unacceptably high. Thus, novel treatment options are required. This dissertation research was undertaken to evaluate a novel therapy to improve health and production in Holstein cows. A series of studies was conducted to: i) Evaluate the effect of recombinant bovine interleukin-8 (**rbIL-8**) on uterine health and milk production, ii) Evaluate the effect of rbIL-8 treatment on insulin resistance in Holstein calves, iii) Evaluate the effect of rbIL-8 treatment on dry matter intake and orchestrated homeorhetic changes that prioritize milk production in Holstein cows, iv) Evaluate associations between periparturient plasma insulin concentration and milk production of Holstein cows, and v) Evaluate the effects of treating Holstein cows with pegylated recombinant bovine granulocyte colony stimulating factor (**rbG-CSF**) on periparturient diseases, milk production, and reproductive performance.

Chapter 2 describes two studies evaluating the effects of rbIL-8 administered via intrauterine (**IU**) infusion shortly after parturition on uterine health, lactation performance, and blood metabolites. Recombinant bIL8-IU treatment was effective in preventing puerperal metritis in multiparous cows, reducing the incidence of postpartum hyperketonemia (**HYK**), and increasing milk production in the long-term.

Chapter 3 describes the effect of systemic administration of rbIL-8 to Holstein calves on peripheral tissue insulin sensitivity. Recombinant bIL-8 induced insulin resistance accompanied by systemic inflammation and caused alterations to blood metabolites and white blood cell populations.

In Chapter 4 we evaluated whether recombinant bovine interleukin-8 treatment administered intrauterine or intravenously within 12 h of parturition would increase milk production through effects on insulin resistance, dry matter intake, and/or by altering metabolism. Findings from Chapter 4 reinforce our findings on increased milk production following rbIL-8-IU treatment. Intrauterine administration of rbIL-8 increased dry matter intake and did not alter metabolism. Treatment with rbIL-8-IU reduced the incidence of HYK and improved cow overall health during the postpartum period.

In Chapter 5, we assessed the association between plasma insulin concentration around parturition and milk yield. Chapter 5 highlights the importance of suppression of postpartum insulin secretion as a key endocrine adaptation to support high milk production.

Chapter 6 describes a study evaluating the effects of treating Holstein cows with rbG-CSF on periparturient diseases, milk production, and reproductive performance. Although rbG-CSF treatment increased circulating white blood cell counts, it was ineffective in improving health and reproduction. In fact, the incidence of lameness and combined disease morbidity was increased for cows treated with rbG-CSF.

In summary, this dissertation provides new insights into a novel therapy to improve health and lactation performance in Holstein cows. We conclude that IU administration of rbIL-8 shortly after parturition is an effective therapy to improve heath and lactation performance in lactating cows. More studies are needed to elucidate the exact mechanism by which rbIL8-IU treatment increases dry matter intake. We do not support the use of rbG-CSF treatment to prevent periparturient diseases in Holstein cows. Further research is needed to confirm the negative effects of rbG-CSF treatment on postpartum lameness.

BIOGRAPHICAL SKETCH

Martin Hugo Zinicola was born in Santa Fe, SF, Argentina. He is the son of Hugo Genaro Zinicola and Adriana Teresita Rossi. He has three sisters, Cecilia Alejandra Zinicola, Marina Belen Zinicola, and Laura Sofia Zinicola.

His father owns a beef cattle operation, and Martin enthusiastically participated in the care and management of the animals since he was a small child. Those formative years stoked Martin's strong interest in food-animal production, which in turn led to his attendance at the National University of Litoral (Faculty of Veterinary Medicine, Esperanza, SF, Argentina) where he earned a degree in veterinary medicine. During his early career, Martin had the good fortune to be mentored by Professor Oscar Jorge Garnero, who instilled in him the passion and professionalism that Martin now demonstrates as a veterinarian. As a veterinary student, Martin had the joy of meeting his future wife, Valeria Tocci, who has been a constant source of love, support and encouragement throughout Martin's career, and is the foundation of the young family they have nurtured together in Ithaca, New York, blessed with two amazing sons, Martino and Benjamin Zinicola, and an unborn baby.

After graduation, Martin's passion for the dairy industry took him to Otautau, New Zealand, where he worked as an assistant herd manger in a large dairy operation – without knowing a single word of English! Martin's attendance at ~1,500 calvings in a muddy, wet environment and very cold weather, significantly enriched both his life experience and his veterinary skills. After he returned to Argentina, Martin worked as a veterinary practitioner for three years. During that time he graduated with a Buiatric specialist degree from National University of Litoral (Faculty of Veterinary Medicine, Esperanza, SF, Argentina).

In 2013, good fortune struck once again when Martin crossed paths with Rodrigo Bicalho, who made possible Martin's journey to Cornell University. Martin first completed the Ambulatory and Production Medicine residency program at Cornell University. As a resident, Martin identified his passion for research, and took the opportunity to pursue his PhD work in the Department of Animal Science, Cornell University.

This dissertation is dedicated to my wife Valeria and my sons Benjamin and Martino for they love and support.

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LIST OF ABBREVIATIONS

BCS,	body condition score
BHB,	β-hydroxybutyrate
bST,	bovine somatotropin
BW,	body weight
CK,	clinical ketosis
CSF,	colony-stimulating factors
CXCR,	CXC receptor
DIM,	days in milk
DMI,	dry matter intake
ECM,	energy corrected milk
ERK,	extracellular signal-regulated kinase
FCM,	fat corrected milk
G-CSFR,	granulocyte colony stimulating factor receptor
GH,	growth hormone
GPCRs,	G-protein coupled receptors
HOMA-IR,	homeostatic model assessment of insulin resistance
HYK,	hyperketonemia
IGF-1,	insulin-like growth factor-I
IL,	interleukin
IU,	intrauterine
IV,	intravenous
IVGTT,	intravenous glucose tolerance test
IVICT,	intravenous insulin challenge test
LPS,	Lipopolysaccharide
MOP,	myeloperoxidase
NAP-1,	neutrophil-activating peptide
NF-κB,	nuclear factor kappa-light-chain-enhancer of activated B cells
PEG,	pegbovigrastim
PUN,	plasma urea nitrogen
QUICKI,	quantitative insulin sensitivity check index
rbG-CSF,	recombinant bovine granulocyte colony stimulating factor
rbIL-8,	recombinant bovine IL-8
RFM,	retained fetal membranes
RQUICKI,	revised QUICKI
$RQUICKI_{BHB,}$	revised QUICKI including BHB
RT,	rectal temperature
TG,	triglycerides
TNF-α,	tumor necrosis factor-alpha

CHAPTER 1: INTRODUCTION

THE NEED FOR NOVEL THERAPIES IN PERIPARTURIENT DAIRY COWS

The transition period, defined as the period from 21 days before to 21 days after parturition, is extremely important and challenging for dairy cows (Drackley, 1999). High-producing dairy cows undergo extreme metabolic adaptations during this period. Feed consumption increases approximately 2-fold between the week preceding parturition and the first 30 days postpartum; however, nutrient intake is often insufficient to meet lactation demands (Bell, 1995; Reynolds et al., 2003). During this period of negative nutrient balance, homeorhetic mechanisms trigger the mobilization of body reserves to support milk synthesis. Transient insulin resistance allows for glucose to be spared by peripheral tissues and directed toward synthesis of lactose in the mammary gland. Furthermore, reduced insulin response enhances lipolysis and muscle breakdown (De Koster and Opsomer, 2013). As a result of this catabolic state, high-producing dairy cows might lose 1.5 kg/day of body weight in the first three weeks postpartum when energy balance is -7 to -9 Mcal NEL/day (Bell, 1995; Koltes and Spurlock, 2011). The liver is the major organ responsible for processing nutrients and metabolites during the transition period (Reynolds et al., 2003). Thus, controlling homeorhesis and optimizing hepatic function early postpartum is expected to enhance lactation performance.

As a result of increased lipolysis, the transition period is characterized by increased circulating fatty acids and β-hydroxybutyrate (**BHB**). Numerous experiments have suggested that a period of severe negative energy balance, imposed by the rapidly escalating energy demands to support milk synthesis and body maintenance, is likely to impair health and reproductive performance of lactating dairy cows (Butler et al., 2003; Ospina et al., 2010; McArt et al., 2013). An association between a greater degree of negative energy balance (elevated pre and/or postpartum fatty acids concentration and post-partum BHB concentration) and decreased neutrophil function (developed around the time of calving, including decreased chemotaxis, phagocytosis, and killing ability in high-producing dairy cows) has been reported in several previous studies that compared cows that developed postpartum infectious diseases with healthy

cows (Cai et al., 1994; Kimura et al., 1999; Kimura et al., 2002; Hammon et al., 2006; Galvao et al., 2010).

Postpartum uterine diseases have been shown to have a detrimental effect on reproductive performance, milk production, and survivability in dairy cattle (Gilbert et al., 2005; Overton and Fetrow, 2008; Dubuc et al., 2011). Many studies have identified and evaluated risk factors associated with uterine diseases, including such factors as BCS, dystocia, twinning, stillbirth, vaginal tear, feed intake, uterine contamination with bacteria shortly after parturition, and impaired uterine defense mechanisms (Kimura et al., 2002; Dubuc et al., 2010; Bicalho et al., 2012; Vieira-Neto et al., 2016). Retained fetal membranes (**RFM**) is defined as the failure to expel the placenta within 24 h after calving (Kelton et al., 1998). It has been hypothesized that RFM might be caused by an impaired immune function during the peripartum period, with a special emphasis on neutrophil function and migration. Thus, the failure of placental detachment seems to be, at least in part, due to a reduced ability of neutrophils to digest the cotyledon-caruncle attachment after parturition.

The relationship between uterine diseases and decreased migration and phagocytic activity of leukocytes has been well studied. It has been demonstrated that neutrophils from cows that developed RFM had a decreased response to chemoattractants and reduced ingestion capacity through the transition period (Gunnink, 1984a; b; c). Moreover, Kimura et al. (2002) demonstrated that neutrophils of cows affected with RFM had decreased myeloperoxidase activity and chemotactic properties and, importantly, these differences were detected up to 10 days before parturition (Kimura et al., 2002). A recent study evaluated the proportion of neutrophils in the uterine lumen during the early postpartum and its associations with uterine bacterial infection and reproductive performance (Gilbert and Santos, 2016). In that study, cows with the greatest influx of neutrophils into the uterus in the immediate postpartum period were associated with improved uterine health and reproductive performance. Additionally, cows free of intrauterine bacteria on the day of calving had more intrauterine neutrophils compared to

infected cows. Thus, a greater influx of neutrophils into the uterus in the immediate postpartum period seems to be critical for the prevention of postpartum uterine diseases.

Puerperal metritis occurs within 21 d postpartum and is defined as an inflammatory process of the uterus characterized by fetid, red-brown, and watery uterine discharge associated with signs of systemic illness and rectal temperature (**RT**) > 39.5 °C. When systemic signs are not present and RT ≤ 39.5, the condition may be defined as clinical metritis (Sheldon et al., 2006). Clinical and puerperal metritis are associated with reduced milk production, poor reproductive performance, high treatment cost and increased risk of culling, resulting in significant economic losses (Huzzey et al., 2007; Overton and Fetrow, 2008; Wittrock et al., 2011). Endometritis is defined as inflammation of the endometrium 21 days after parturition without systemic signs of illness, and it is characterized by the presence of mucopurulent uterine discharge detectable in vagina (Sheldon et al., 2006). It is well known that endometritis impairs reproduction (Machado et al., 2015), and thus affecting the economy of the dairy industry.

Uterine diseases are highly correlated with bacterial contamination and immune suppression (Dubuc et al., 2010; Bicalho et al., 2012). Thus, recruitment of neutrophils to the uterine lumen seems to be a key factor for early clearance of bacterial contamination (Kimura et al., 2002; Hammon et al., 2006).

Mastitis is a highly prevalent disease in dairy cows and arguably the most important disease for the dairy industry worldwide, causing economic losses due to reduced milk production, discarded milk, lower conception rates, premature culling, and treatment costs (Bar et al., 2008; Hertl et al., 2010; Cha et al., 2011). The well-documented reduction in milk production resulting from mastitis is estimated at approximately 15% of the milk production potential of the affected cow (Seegers et al., 2003; Bar et al., 2008; Schukken et al., 2009). Clinical mastitis is also a serious animal welfare issue as it is associated with pain, reduced well-being, and behavioral changes (Medrano-Galarza et al., 2012).

The healthy mammary gland has a resident leukocyte population and healthy milk contains up to 100 somatic cells, 75 % of which are leukocytes and between 3% and 25% are

polymorphonuclear cells. However, neutrophils comprise up to 90% of total milk leukocytes in mastitic milk (Ezzat Alnakip et al., 2014). Neutrophils are the first defense cell line to arrive in the mammary gland once pathogen invasion occurs. They enter the mammary gland environment from the blood stream through adhesion to the endothelium and diapedesis towards the lumen of the mammary alveoli. Their principal role is phagocytosis and killing of invading bacteria, preventing microbial multiplication within the mammary gland. Therefore the ability of the mammary gland to counteract bacterial infection relies mainly on: (1) circulating neutrophils and their ability to enter the alveoli lumen, and (2) their phagocytic and killing capacity (Ezzat Alnakip et al., 2014). Additionally, it is well known that the mammary gland is an excellent environment for microbial growth, given the availability of nutrients, temperature and humidity that are optimum for bacterial growth. Therefore, the cow's immune system has to establish a proper and fast immune response to effectively clear the bacterial infection.

Despite decades of research dedicated to advancing knowledge and aiding in the prevention of periparturient disorders (e. g. mastitis, retained placenta, and metritis) their incidences are still high (Ribeiro et al., 2013). Moreover, these infectious diseases are frequently treated with systemic or local antibiotic therapy (Pol and Ruegg, 2007; Lima et al., 2014). The emergence of antibiotic-resistant bacteria is a mounting concern, since many of the antibiotics used in animal agriculture can also be used in human medicine (Silbergeld et al., 2008). Considering this, there is growing demand for a substitute to antibiotic use and for more effective treatment options that could improve health in dairy cows. Therefore, in this dissertation we evaluated the use of a potential alternative, recombinant cytokines, which could be used to improve health, production, and reproduction in lactating cows.

CYTOKINES

Cytokines are a large group of signaling proteins that regulate a wide range of biological processes. The main function of cytokines is to mediate and regulate immunity; however, in the

last decades, the list of functions attributed to cytokines has expanded. Today, it is known that cytokines are involved in the regulation of cell proliferation, hematopoiesis, angiogenesis, inflammation, tissue repair, and others biological processes (Nagata, 1989; Goldring and Goldring, 1991; Onuffer and Horuk, 2002; Turner et al., 2014). Cytokines are produced by several cell types in the body and act on the cells that secreted them (autocrine action), act on nearby cells (paracrine action), or may reach the circulation to act on target cells at distant body sites (endocrine action) (Zhang and An, 2007). Cytokines have been classified into three broad categories according with their role on inflammation as pro-inflammatory cytokines, anti-inflammatory cytokines, and chemokines (Zhang and An, 2007). Pro-inflammatory cytokines are produced mainly by macrophages and are key regulators of inflammatory reactions. Major pro-inflammatory cytokines include tumor necrosis factor-alpha (**TNF-α**), interleukin-6 (**IL-6**), IL-2, IL-1β, and interferon gamma (Dinarello, 2000). Conversely, anti-inflammatory cytokines act to reduce inflammation and promote healing. Major anti-inflammatory cytokines include IL-10, IL-4, IL-11, and IL-13 (Opal and DePalo, 2000). Chemokines stimulate migration and activation of leukocytes. Until today, more than 50 chemokines have been identified, and the major studied chemokines are IL-8, monocyte chemoattractant protein 1 (**MCP-1**), and macrophage inflammatory protein-1alpha (**MIP-1α**) and MIP-1β (Graves and Jiang, 1995). Others, however, have grouped cytokines based on their actions such us growth factors (e.g. transforming growth factor beta), colony-stimulating factors (e.g. granulocyte-colony stimulating factor, **G-CSF**), and chemoattractant proteins (e.g. IL-8 and MCP-1; Dinarello, 2007).

In this introduction we describe the main features of the two cytokines studied in this dissertation, G-CSF and IL-8, as well as their potential use as a therapy to improve health and production in Holstein cows.

Granulocyte-colony stimulating factor (G-CSF)

In 1960, the development of *in vitro* techniques for the clonal culture of hematopoietic cells in semisolid culture medium allowed the discovery of several colony-stimulating factors

(**CSF**). In those assays, precursor cells from bone marrow proliferate to form colonies in response to a specific colony-stimulating factor (Nagata, 1989). Later, the advance in technology and science showed that there were in fact several types of CSFs. One of the CSFs that has been well studied is G-CSF. In 1983, G-CSF was first recognized and purified in mice and cloned a few years later (Metcalf, 1985; Nagata et al., 1986). Granulocyte CSF is an endogenous hematopoietic growth factor (19 kDa in size and composed of 174 amino acid residues) that stimulates the production and differentiation of neutrophils by progenitor cells in the bone morrow (Clark and Kamen, 1987; Nagata, 1989). Moreover, it has been demonstrated that the antimicrobial function of mature neutrophils is enhanced by G-CSF treatment (Roberts, 2005).

All cellular responses to G-CSF occur as a consequence of the activation of a specific transmembrane receptor located on the surface of responsive cells (**G-CSFR**). The G-CSFR receptor is expressed in neutrophils and their precursors (Nicola and Metcalf, 1985; McKinstry et al., 1997), monocytes (Christopher et al., 2011), platelets (Shimoda et al., 1993), activated T and B-lymphocytes (Franzke et al., 2003), endothelial cells (Bussolino et al., 1989), placental cells (Uzumaki et al., 1989), and trophoblastic cells (Uzumaki et al., 1989). Upon G-CSF binding to its receptor, the activated G-CSFR triggers a series of intracellular signaling events; activates the Janus tyrosine kinases (**JAK** 1 and 2), signal transducers and activators of transcription (**STAT**) proteins, and mitogen-activated protein kinases (**MAPK**) signaling pathways (Avalos, 1996; Roberts, 2005; Marino and Roguin, 2008). As a result of the activation of these signaling pathways, G-CSF influences cell survival, differentiation, and proliferation.

The invention of recombinant DNA (rDNA) technologies in the early 1970s has permitted the production of human rhG-CSF (Souza et al., 1986; Zsebo et al., 1986). Because of the ability of G-CSF to increase numbers and function of neutrophils, extensive investigations have evaluated the use of rG-CSF in humans and cattle as a therapeutic drug. Today, there are three forms of rG-CSF commercially available to be used in humans (filgrastim, pegfilgrastim, and lenograstim) and one form to be used in cattle (pegbovigrastim, **PEG**).

In humans, the use of rhG-CSF has been demonstrated to be effective in reducing the incidence of febrile neutropenia, a frequent event observed in cancer patients treated with chemotherapy and characterized by neutrophil numbers below normal values (Cooper et al., 2011). For instance, patients with breast cancer receiving chemotherapy and treated with pegfilgrastim had lower incidence of febrile neutropenia, lower incidence of febrile neutropenia-related hospitalization, and lower use of intravenous anti-infective drugs when compared with placebo patients (Vogel et al., 2005). Moreover, the use of G-CSF has been indicated to treat acute myeloid leukemia (**AML**; Lowenberg et al., 2003). However, studies evaluating the effect of G-CSF on patients with AML have yielded conflicting results. A study has suggested that G-CSF treatment may accelerate disease progression in a small subset of patients suffering AML (Basnett et al., 2017). Furthermore, G-CSF treatment has been recommended as a therapy for severe chronic neutropenia (Bonilla et al., 1994; Cottle et al., 2002). Interestingly, some studies have suggested the use of G-CSF in women with recurrent pregnancy loss as treatment option (Cavalcante et al., 2015) . However, more studies are needed to confirm the beneficial effects of G-CSF administration on embryonic survival.

In cattle, the use of PEG (Imrestor, Elanco Animal Health) in periparturient dairy cows was shown to increase the concentrations of circulating neutrophils (Kimura et al., 2014; Canning et al., 2017; McDougall et al., 2017). However, discrepant results have been reported for monocytes and lymphocytes concentrations. Kimura et al. (2014) observed no differences between PEG-treated and untreated cows in terms of monocytes and lymphocytes numbers. In contrast, a study by McDougall et al. (2017) showed a significant increase in monocytes and lymphocytes counts. A recent study showed that the administration of PEG in cows was associated with higher eosinophil numbers (Van Schyndel et al., 2018). Moreover, it has been shown that pegbovigrastim treatment enhanced *in vitro* myeloperoxidase (**MOP**) release from neutrophils of periparturient cows (McDougall et al., 2017). However, neutrophil phagocytic activity, oxidative burst, and MOP function did not differ between treated and untreated cows (McDougall et al., 2017). Similar results were observed in a previous study (Kimura et al.,

2014). Thus, it is unclear if the antimicrobial properties of neutrophils could be enhanced with the use of PEG in periparturient dairy cows.

Few studies have demonstrated efficacy in the prevention of clinical mastitis with the use of PEG (Hassfurther et al., 2015; Canning et al., 2017; Ruiz et al., 2017). In the study by Hassfurther et al. (2015), healthy cattle received subcutaneous injections of sterile saline (0.9% NaCl) solution (control treatment) or PEG at 5, 10, or 20 μg/kg at approximately 7 days before the anticipated date of parturition. Cattle treated with PEG at 10 and 20 μg/kg had significantly fewer cases of clinical mastitis (9/54 and 5/53, respectively), compared with control cattle (18/53). Furthermore, Canning et al. (2017) evaluated the effect of PEG on postpartum CM incidence from 4 dairy farms located in different states of the United States. They observed that treated cows were associated with an overall (across all dairies) 35% reduction of CM incidence compared with controls. Lastly, a reduction in CM incidence of 25% was reported in cows treated with PEG relative to controls (Ruiz et al., 2017).

Furthermore, in the study conducted by Ruiz et al. (2017), cows that received PEG had a 5.8% greater chance of being inseminated within the first 100 DIM postpartum, and Canning et al. (2017) reported a 52% reduction in failure to return to estrus by 80 DIM in cows treated with PEG, relative to control cows.

Interestingly, it was reported that cows diagnosed with metritis and treated with PEG produced 2.3 kg/d more milk than control cows with metritis (Ruiz et al., 2017). Moreover, the same study showed that cows with clinical mastitis and treated with PEG produced 2.1 kg/d more milk than control cows with mastitis. However, the exact mechanism by which PEG treatment increases milk production in sick animals remains unknown.

Therefore, the administration of PEG in periparturient dairy cows increases leukocytes numbers and seems to be a good treatment option to improve health and reproductive performance. However, more clinical trials are needed to confirm the beneficial effects of PEG administration in periparturient dairy cows.

Interleukin-8

The term “Interleukin” was first proposed in 1979 (Mizel and Farrar, 1979). The term interleukin derives from “inter” as a mean of their ability to communicate between different population of leukocytes, and “leukin” because many of these proteins are produced and act on leukocytes. Interleukine-8 is a chemokine that has a molecular size of 11 kDa and is composed of 72 amino acid residues. It was first described in 1987-88 and named as neutrophil-activating peptide (**NAP-1**; Yoshimura et al., 1987; Van Damme et al., 1988). However, a further study discovered that NAP-1 also chemoattracted a subset of T-lymphocytes and they therefore proposed to rename NAP-1 as IL-8 (Larsen et al., 1989).

Interleukin-8 belongs to a family of cytokines in which two of the conserved N-terminal cysteine (C) residues are separated by another amino acid, C-X-C (Holmes et al., 1991). CXC chemokines, such us IL-8, attract and activate neutrophils. Whereas CC chemokines, such as regulated on activation normal T cell expressed and secreted (RANTES) and monocyte chemotactic protein-1, activate eosinophils, basophils, and T-lymphocytes (Graves and Jiang, 1995; Crawford et al., 2011). Interleukin-8 is produced by smooth muscle cells, epithelial cells, endothelial cells, and cells of the innate immune system with toll-like receptors (Kaplanski et al., 1994; Mitchell et al., 2003). It has been demonstrated that IL-8 acts via the activation of two receptors, named CXC chemokine receptor 1 and 2 (**CXCR1** and **CXCR2**), located in the surface of responsive cells (Holmes et al., 1991). CXC chemokine receptors 1 and 2 belong to the family of G-protein coupled receptors (**GPCRs**); these receptors have seven transmembrane domains (Rosenbaum et al., 2009). CXC chemokine receptor 1 is highly specific for IL-8. However, CXCR2 binds to IL-8 as well as other CXC chemokines containing the presence of a sequence motif (Glu-Leu-Arg), named ELR (Ahuja and Murphy, 1996; Onuffer and Horuk, 2002). To the best of my knowledge, CXCR1 and CXCR2 receptors expression has been detected in neutrophils (Hammond et al., 1995), macrophages (Morohashi et al., 1995), T-lymphocytes (Morohashi et al., 1995), eosinophils (Petering et al., 1999), basophils (Ochensberger et al., 1999), mast cells (Nilsson et al., 1999), dendritic cells (Sozzani et al.,

1997), neurons and astrocytes of the brain (Horuk et al., 1997; Puma et al., 2001), hepatocytes (Kuboki et al., 2008; Clarke et al., 2011), adipocytes (Gerhardt et al., 2001), and mammary alveolar epithelial cells (Siebert, 2013).

Interleukin-8 has two primary functions. It induces chemotaxis of neutrophils, causing them to arrive to the site of infection, and it stimulates neutrophil activation (Mitchell et al., 2003). The CXCRs activation stimulates the α subunit of the G-protein to exchange its bound guanosine diphosphate (GDP) for a guanosine triphosphate (GTP). With this GTP bound the G-protein is in an active state. The activated G-protein dissociates into the α subunit (Gq), and a β-γ complex. The activated α subunit activates the enzyme phospholipase C (**PLC**). This enzyme acts on the molecule phosphatidylinositol 4,5-bisphosphate (PIP2). Phospholipase C cleaves PIP2 into two molecules: inositol 1,4,5 triphosphate (IP3) and diacyglycerol (**DAG**). Then, IP3 travels to the endoplasmic reticulum and triggers an increase of calcium (**Ca**) flux to the cytosol. At the same time that IP3 is initiating Ca release, DAG activates protein kinase C (**PKC**). Calcium is necessary for fully activity of PKC. Once activated, PKC phosphorylates a number of proteins responsible of leukocyte activation (Bokoch, 1995; Tharp et al., 2006). Moreover, studies haven shown that PKC activation and increased Ca levels are not the only factors responsible for leukocyte activation. The activation of CXCRs also stimulate the MAPK/ERK pathway and activates low-molecular-weight GTP-binding proteins (Bokoch, 1995). Thus the exact mechanisms by which IL-8 stimulate neutrophil activation, respiratory burst, vesicle secretion, and cytoskeletal assembly and motility remains under investigation.

Studies with human and rat hepatocytes showed that IL-8 reduced apoptosis and stimulated cell proliferation *in vitro* (Colletti et al., 1998; Osawa et al., 2002). For instance, antibody neutralization of CXC chemokines impaired liver regeneration in rats subjected to 70% hepatectomy (Colletti et al., 1998). Moreover, mice treated with rIL-8 and ConA-induced liver damage resulted in lower transaminases activities (AST and ALT) and significantly reduced liver damage (Osawa et al., 2002). Although the exact mechanism by which IL-8 reduced cell apoptosis is unclear, it was demonstrated that IL-8 delayed neutrophil apoptosis by activating

extracellular signal-regulated kinase (**ERK**) and phosphoinositide 3-kinase (Klein et al., 2000). Furthermore, IL-8 also promotes angiogenesis trough the stimulation of a potent vasculogenic and angiogenic factor, named vascular endothelial growth factor (**VEGF**), by the activation of different signaling pathways (Strieter et al., 1995; Martin et al., 2009; Hou et al., 2014).

The activation of nuclear factor kappa-light-chain-enhancer of activated B cells (**NF-κB**) during mammary gland development is critical for mammary cell differentiation (Brantley et al., 2000; Brantley et al., 2001; Cao et al., 2001). Prolactin and progesterone are the primary hormones necessary to ensure proper mammary gland development during pregnancy. Interestingly, it has been demonstrated that both hormones induced the expression of receptor activator of NF-κB ligand (RANKL), which is essential to activate NF-κB and up-regulate cyclin D1 (Srivastava et al., 2003). Interestingly, IL-8 has the ability to activate NF-κB trough TRAF6 and Rho-GTPase pathways (Manna and Ramesh, 2005). Interleukine-8 is also known as an activator of RANKL (Bendre et al., 2003).

Results from the human literature indicate that IL-8 is particularly important for the development of insulin resistance. The expression of IL-8 in omental fat depots is 2.7-fold greater in obese humans that are insulin-resistant compared with obese patients classified as insulin-sensitive (Hardy et al., 2011). The direct effect of IL-8 on the response to insulin was demonstrated *in vitro*, wherein exposure to IL-8 induced insulin resistance in human adipocytes (Kobashi et al., 2009). Accordingly, previous studies indicate that IL-8 plays an important role in tissue mobilization during feed restriction. Obese men subjected to a low-calorie diet lost 15% of body weight and 30% of body fat in 24 weeks (Bruun et al., 2003). As opposed to other adipokines, which concentrations in blood decreased concurrently with the reduction in body fat, IL-8 concentrations increased by 30% and were not correlated with reestablishment of insulin sensitivity. The aforementioned data are in agreement with the metabolic milieu observed in animals suffering from infectious diseases. Stimulation of toll-like receptors using bacterial particles induced expression of IL-8, impaired insulin-mediated glucose transport, stimulated lipolysis, and led to insulin resistance (Franchini et al., 2010); a mechanism that ensures glucose

availability to support immune function. The mechanism by which IL-8 promote insulin resistance seems to be via the inhibition of AKT activation through MAPK/ERK pathways (Kobashi et al., 2009).

Our group has developed a recombinant bovine IL-8 (**rbIL-8**) molecule from bacterial culture with the original objective of improving postpartum uterine health (Bicalho et al. 2018). Our group has conducted a series of experiments to evaluate the safety of rbIL-8 administration and to assess its biological activity *in vitro* and *in vivo*. Results from *in vitro* chemotactic assays confirmed the bioactivity of rbIL-8. Based on optical density values, chemoattractant properties of rbIL-8 was 10-fold greater compared with control wells. Two *in vivo* studies were conducted to assess the safety and the biological activity of rbIL8. For study 1, one-year-old Holstein heifers were randomly allocated to receive a single intravaginal infusion containing 1,125 μg of rbIL-8 diluted in 20 mL of saline solution or a single intravaginal infusion of 20 mL of saline solution. Similar to *in vitro* results, a significant increase in vaginal neutrophils was observed in heifers treated with rbIL-8 within 3 h of treatment, but not in control heifers. For study 2, non-pregnant lactating Holstein cows were randomly allocated to receive an intrauterine infusion with 1,125 μg of rbIL-8 diluted in 20 mL of saline solution, a positive control consisting of resin-purified lysate of *E. coli* BL21 not transfected with the plasmid coding for rbIL-8 diluted in 20 mL of saline solution, and a negative control infused with 20 mL of saline solution. Intrauterine infusion of rbIL-8 increased the proportion of neutrophils in uterine cytological samples from 3.5% before treatment to 75.8% 24 h later – an increase that was not observed in untreated controls and cows treated with resin-purified lysate of *E. coli* BL21 that was not transfected with the plasmid coding for rbIL-8 (Bicalho et al., 2018). In summary, our group demonstrated that our rbIL-8 molecule elicited the expected biological responses; strong chemoattractant properties *in vitro* and *in vivo* (following intravaginal and intrauterine infusions). More importantly, systemic, intrauterine, and intravaginal administration of rbIL-8 did not result in detectable undesirable side effects such us fever, increased respiratory rate, dehydration, and/or signs of pain and discomfort.

Kimura et al. (2002) studied plasma IL-8 concentrations in cows with and without RFM from 15 days prior to parturition to 15 days post parturition and observed that cows that did not develop RFM had greater plasma IL-8 concentrations, both before and after calving. Thus, the presence of an effector molecule such as IL-8 to attract neutrophils into the uterus seems essential for maintenance of uterine health.

Therefore, the intrauterine administration of rbIL-8 shortly after parturition has the potential to recruit and activate neutrophils into the uterus, resulting in early influx of neutrophils into the uterine lumen, early detachment of the placenta, early bacterial contamination clearance, and ultimately healthier cows.

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CHAPTER 2: Effects of recombinant bovine interleukin-8 (rbIL-8) treatment on health, metabolism, and milk production in Holstein cattle II: Postpartum uterine health, hyperketonemia, and milk production

ABSTRACT

To evaluate the effect of recombinant bovine interleukin-8 (rbIL-8) on uterine health and milk production two separate studies were conducted. The first study was originally designed to investigate the effect of intrauterine administration of rbIL-8 on the prevention of postpartum uterine diseases; an unexpected increase in milk production was observed as a result of intrauterine rbIL-8 administration. To further validate the effect of rbIL-8 administration on milk production a second study was conducted; study 2. For study 1, postpartum Holstein cows (n = 213) were randomly allocated into one of three intrauterine treatment groups: control (CTR, 250 mL of saline solution), low-dose (L-IL8, 11.25 µg of rbIL-8 diluted in 250 mL of saline solution), and high-dose (H-IL8, 1,125 µg of rbIL-8 diluted in 250 mL of saline solution). Intrauterine delivery of treatments was performed within 12 h of parturition. Cows were evaluated for retained fetal membranes, puerperal metritis, and clinical endometritis. Blood samples were collected immediately before treatment and 1, 2, and 3 DIM for assessment of IL-8, haptoglobin, fatty acids, and β-hydroxybutyrate concentrations. Treatment with rbIL-8 reduced the incidence of puerperal metritis in multiparous cows (CTR = 34.3, L-IL8 = 8.11, and H-IL8 = 6.35%). Both, the L-IL8 and H-IL8 groups produced significantly more milk, FCM, and ECM yields when compared with placebo treated controls. Moreover, rbIL-8 treatment significantly decreased the incidence of postpartum hyperketonemia (**HYK**; CTR = 35.3, L-IL8 = 15.8, and H-IL8 = 22.2%). A second study was performed to confirm the effect of rbIL-8 on milk production. In study 2, 164 primiparous cows were randomly allocated into one of four treatment groups: control (CTR, 250 mL of saline solution), low-dose (L-IL8, 0.14 µg of rbIL-8), medium-dose (M-IL8, 14 µg of rbIL-8), and high-dose (H-IL8, 1,400 µg of rbIL-8). Treatments were prepared and administered as described for study 1. Cows in the L-IL8, M-IL8, and H-IL8 groups produced significantly more milk, FCM, and ECM yields when compared to control cows. In conclusion, treatment with rbIL-8 decreased the incidence of HYK and puerperal metritis in multiparous cows. The administration of rbIL-8 was repeatedly associated with a dramatic and long-lasting improvement of lactation performance.

Keywords: Interleukin-8, metabolism, milk production

INTRODUCTION

Postpartum uterine diseases such as metritis, endometritis, and retained fetal membranes (**RFM**) are extremely relevant due to animal welfare and economic reasons. Such diseases are often painful and cause discomfort, increase costs with treatments, reduce milk production and reproductive efficiency, and increase the risk of culling (Gilbert et al., 2005, Han and Kim, 2005, Overton and Fetrow, 2008). Metritis and endometritis are commonly associated with mixed bacterial infection of the uterus, including by *Escherichia coli*, *Trueperella pyogenes*, and *Fusobacterium necrophorum* (Bicalho et al., 2012). A physiological factor contributing to the increased susceptibility to uterine disease is the natural immunosuppression experienced by cows during the periparturient period (Drackley, 1999, Hammon et al., 2006, Galvao et al., 2010).

Neutrophils are the main leukocyte involved in the release of fetal membranes as well as in bacterial clearance after uterine infection (Hussain, 1989, Kimura et al., 2002). Neutrophil function begins to decline prior to parturition, reaches a nadir shortly after parturition, and slowly returns to prepartum levels by about 4 weeks postpartum (Kehrli and Goff, 1989, Goff and Horst, 1997). Several factors contribute to this decline in neutrophil function including an increase in blood estradiol and cortisol concentrations around calving and nutrient deficiencies such as vitamins A and E, calcium, and selenium (Goff and Horst, 1997, Kimura et al., 2002, Hammon et al., 2006). In fact, neutrophils from cows with RFM have decreased migration ability and impaired myeloperoxidase activity (Kimura et al., 2002). On the other hand, cows with greater neutrophil influx to the uterus are less susceptible to bacterial infections and are at a lower risk of developing endometritis (Gilbert et al., 2005).

Interleukin-8 (**IL-8**) is a pro-inflammatory cytokine and the main chemoattractant for neutrophils. It is produced by smooth muscle, epithelial cells, endothelial cells, and cells of the innate immune system with toll-like receptors (Mitchell et al., 2003). Binding of IL-8 to its receptors CXCR1 and CXCR2 on neutrophil surface induces neutrophil activation, stimulates

chemotaxis, and increases phagocytosis and killing ability (Mitchell et al., 2003). Kimura et al. (2002) studied plasma IL-8 concentrations in cows with and without RFM from 15 days prior to parturition to 15 days post parturition and observed that cows that did not develop RFM had greater plasma IL-8 concentrations, both before and after calving. Thus, the presence of an effector molecule such as IL-8 to attract neutrophils into the uterus seems essential for maintenance of uterine health.

Our group has developed a recombinant bovine IL-8 (**rbIL-8**) molecule with the original objective of improving postpartum uterine health (Bicalho et al., 2018). We conducted a series of experiments to evaluate the safety of rbIL-8 administration and to assess its biological activity *in vitro* and *in vivo*. We demonstrated that our rbIL-8 molecule elicited the expected biological responses; strong chemoattractant properties *in vitro* and *in vivo* (following intravaginal and intrauterine infusions). More importantly, systemic, intrauterine, and intravaginal administration of rbIL-8 did not result in detectable undesirable side effects such us fever, increased respiratory rate, dehydration, and/or signs of pain and discomfort. (Bicalho et al., 2018).

Therefore, our original hypothesis was that intrauterine administration of rbIL-8 would reduce the incidence of postpartum uterine diseases. Objectives of the first study were to evaluate the effects of two different doses of rbIL-8 administered by an intrauterine infusion within 12 h of parturition on incidence of RFM, puerperal metritis, and endometritis. Upon completion of this randomized clinical trial we identified an unexpected association of rbIL-8 with an increase in milk production, FCM, and ECM yields when compared with controls. To validate our initial results and further investigate the effects of rbIL-8 on milk production, we conducted a second study (study 2) to evaluate the hypothesis that a single administration of rbIL-8 within 12 h of parturition improves lactation performance in Holstein cows. The objective of study 2 was to validate the association of postpartum intrauterine administration of rbIL-8 with increased milk production.

MATERIAL AND METHODS

Ethics statement

The research protocol was reviewed and approved by the Cornell University Institutional Animal Care and Use Committee (protocol number 2013-0039). The methods were carried out in accordance with approved guidelines.

Farm and management

Two studies were conducted in a large commercial dairy farm located in Cayuga County, NY. The farm milked approximately 3,800 Holstein cows thrice daily in a rotary parlor with integrated milk meters that record individual production at every milking (DeLaval, Tumba, Sweden). Cows were housed in naturally ventilated free stall barns with concrete stalls bedded with manure solids. All cows were offered a TMR and feed was pushed up 8 times a day. Diets consisted of approximately 55% forage and 45% concentrate on a dry matter basis. Diets were formulated to meet or exceed the National Research Council nutrient requirements for lactating Holstein cows weighing 650 kg and producing 45 kg of 3.5% fat-corrected milk (National Research Council, 2001). Cow displaying signs of calving were moved to individual maternity pens for delivery, where trained farm personnel assisted with parturition as needed. After calving, cows were transferred to a postpartum pen where they remained for approximately 40 d. Farm's reproductive management used for the first service a Presynch-Ovsynch protocol in combination with estrus detection (Pursley et al., 1995, Moreira et al., 2001). A Resynch (Fricke et al., 2003) protocol was started in non-pregnant cows 33 ± 3 d after previous inseminations. A voluntary waiting period of 50 d was used. Estrus was detected based only on electronic activity sensors worn around the neck (Alpro, DeLaval, MO).

Study design, treatments, and sample collection

Study 1. In total, 213 cows (primiparous, n = 104; multiparous, n = 109) were enrolled in the study from July 2014 to August 2014. Cows were blocked by parity and, within block,

randomly allocated into one of three treatment groups: control (**CTR**; n = 67), low-dose of rbIL-8 (**L-IL8**; n = 80), and high-dose of rbIL-8 (**H-IL8**; n = 66). Cows allocated to L-IL8 and H-IL8 received an intrauterine infusion of 250 mL of saline solution containing 11.25 and 1,125 μg of rbIL-8, respectively. Control cows received an intrauterine infusion of 250 mL of saline solution. Recombinant bovine IL-8 was produced and purified according to the methods described previously (Bicalho et al., 2018). Treatments were administered by the research team within 12 h of parturition. Cows were restrained in headlock stanchions and the perineal area was cleansed with paper towels and disinfected with 70% ethanol. A sterile gilt AI catheter (Livestock Concepts, Hawarden) attached to a 250 mL saline bag was introduced into the cranial vagina. The catheter was manipulated into the uterus, the tip was exposed to the uterine lumen, and the treatment was infused into the uterus.

Blood samples were collected from 60 cows (20 cows per treatment) on d 0 (before treatment), 1, 2, and 3 relative to calving via coccygeal vein/artery puncture into an evacuated tube without anticoagulant (Becton, Dickinson and Company, Franklin Lakes, NJ). Blood samples were transported to the laboratory on ice and centrifuged at 2,000×g for 15 min at 4ºC, Serum was harvested and frozen at -80°C until assayed. For each cow, body condition scores (**BCS**) was recorded at enrollment and at 35 DIM by a single investigator using a 5-point scale with a quarter-point system, as previously described (Edmonson et al., 1989). Rectal temperature (**RT**) was measured at enrollment and at 3, 6, and 9 DIM using a digital thermometer (GLA M750, GLA Agriculture Electronics, CA). Milk yield was recorded at every milking using on-farm milk meters and monthly averages obtained during the first 6 months postpartum were used for statistical analyses.

Study 2. A total of 164 primiparous cows were enrolled in the study from October 2015 to December 2015. Cows were randomly allocated into one of four treatment groups control (**CTR**; n = 41), low-dose of rbIL-8 (**L-IL8**; n = 41), medium-dose of rbIL-8 (**M-IL8**; n = 41), and high-dose of rbIL-8 (**H-IL8**; n = 41). Cows allocated to L-IL8, M-IL8, and H-IL8 received a single intrauterine infusion of 250 mL of saline solution containing 0.14, 14, and 1,400 μg of

rbIL-8, respectively. Control cows received an intrauterine infusion of 250 mL of saline solution. Treatments were administered within 12 h of parturition as described in study 1. Body condition score was scored at enrollment and at 35 DIM. Rectal temperature was recorded at enrollment. Milk yield was recorded at every milking using on-farm milk meters and monthly averages obtained during the first 6 months postpartum were used for statistical analyses.

Analyses of IL-8 and Metabolites in serum (study 1)

Concentrations of IL-8 in serum was determined using a human IL-8 ELISA kit (R&D Systems Inc., Minneapolis, MN), validated for bovine serum (Shuster et al., 1996). Plasma concentrations of fatty acids (NEFA-C® kit; Wako Pure Chemical Industries, Richmond, VA) and β-hydroxybutyrate (**BHB**; (Williamson and Mellanby, 1974), Sigma-Aldrich, St. Louis, MO) were determined by colorimetric methods. Haptoglobin concentrations were determined using a colorimetric procedure as previously described (Bicalho et al., 2014), and reported as optical density readings at 450 nm of wavelength. Endotoxin levels from purified rbIL-8 was measured by a chromogenic assay (Pierce, Chromogenic Endotoxin Quant Kit, ThermoFisher Scientific,Waltham, MA) following manufacture instructions. Endotoxin levels were < 5 EU/mL (data not shown). A total of 3 mL of purified rbIL-8 was used for treatments. Thus, cows treated with rbIL-8 received a solution containing endotoxin levels under the FDA limit of < 5 EU/kg.

Disease Definitions

Retained fetal membranes assessed by trained farm personnel was defined as failure to deliver fetal membranes by 24 h after calving. Puerperal metritis diagnosis was performed by the researcher and by trained farm personnel. Researcher puerperal metritis was diagnosed based on evaluation of vaginal mucus retrieved using a Metricheck device (Metricheck, SimcroTech, Hamilton, New Zealand). Vaginal discharge was scored using a modified 0 to 5 scale (0 = no secretion material retrieved; 1 = clear mucus; 2 = clear mucus with flecks of pus; 3 = mucopurulent discharge containing < 50% of pus; 4 = mucopurulent discharge containing ≥ 50%

of pus; 5 = watery, red-brown, fetid vaginal discharge). Cows with a score = 5 and with a RT >39.5°C were considered to have puerperal metritis (Sheldon et al., 2006). Farm diagnosed puerperal metritis was performed daily by trained farm employees. Cows were flagged for a physical examination when showing signs of dullness and depression or when a milk deviation of more than 4.5 kg was detected. At the physical examination cows were considered as puerperal metritis when a fetid, watery and red-brown uterine discharge combined with systemic illness was observed. For the purpose of this study, cows diagnosed with puerperal metritis by the research team and/or by farm employees were grouped together and considered positive for puerperal metritis. Furthermore, information regarding puerperal metritis diagnosis was not exchanged between the researchers and farm personnel. Clinical ketosis (**CK**) diagnosis was performed by farm personnel and defined as cows with decreased milk production, low rumen fill, weakness, dullness, depression, and high blood concentration of BHB. Diagnosis of clinical endometritis was performed by research personnel at 35 DIM based on evaluation of vaginal mucus using a Metricheck device. Cows with a score ≥ 3 were considered to have clinical endometritis. Hyperketonemia (**HYK**) was defined as cows with serum BHB concentrations ≥1.2 mmol/L on d 1, 2, or 3.

Statistical analysis

Descriptive statistical analyses were performed using JMP PRO version 12 (SAS Institute Inc., Cary, NC) using the ANOVA function for continuous data and chi-square and Fisher's tests for categorical data. Body condition score loss was assessed using the GLIMMIX procedure of SAS. Continuous data collected over time were analyzed using general linear mixed models with the MIXED procedure of SAS. Normality and homoscedasticity of residuals were assessed using residual plots. Initial statistical models included the fixed effects of treatment, parity, dystocia, stillbirth, BCS at calving, days of gestation at calving, RT at calving, sire predicted transmitting ability for milk yield, age in days at calving, time, and the two-way interaction terms between independent variables. Several covariance structures were tested, and the Akaike information

criterion (**AIC**) was used to select the best model fit. Variables and their respective interaction terms were retained in the model when $P \leq 0.15$. Significances were considered when $P \leq 0.05$ or a trend if $0.05 < P \leq 0.10$. For all models, Tukey's honest significance test for multiple comparisons was used. Data are reported as LSM ± SEM unless otherwise stated.

Categorical variables were analyzed by logistic regression models using the binary distribution of the GLIMMIX procedure of SAS. Initial models included the fixed effects of treatment, parity, dystocia, stillbirth, BCS at enrollment, RT at enrollment, and the interaction term treatment by parity. Variables and their respective interaction terms were retained in the model when $P \leq 0.15$. Dunnett's significance test for multiple comparisons was used. To evaluate the effect of all rbIL-8 treatments combined against the CTR group on the incidence of HYK, a contrast was performed. To evaluate the effect of all rbIL-8 treatments on the incidence of CK, Fisher's exact test was used. Finally, the effect of treatment on reproduction was analyzed by Cox's proportional hazard using the PHREG procedure in SAS. Control groups were used as reference for comparison. Treatment, parity, twin, stillbirth, dystocia, BCS at enrollment, and the interaction treatment by parity were offered to the model as independent variables, and retained when $P \leq 0.15$.

RESULTS

Descriptive Data

Descriptive statistics regarding the number of multiparous and primiparous animals enrolled, BCS at enrollment, RT at enrollment, days carried calf, and the incidence of twins, dystocia, stillbirth, and male calf for studies 1 and 2 are depicted in Table 2.1.

Incidence of uterine diseases, hyperketonemia, and clinical ketosis (study 1)

Treatment did not affect the incidence of RFM (CTR = 4.5, L-IL8 = 2.5, H-IL8 = 4.6%; $P = 0.78$). A significant treatment by parity interaction was observed for puerperal metritis ($P = 0.02$). Multiparous cows treated with rbIL-8 had ($P = 0.03$) lower incidence of puerperal metritis

when compared with control cows, and no differences were observed between treatment groups for primiparous cows (Table 2.2). Treatment did not affect RT during the first 9 d postpartum (Figure 2.1). Intrauterine infusion of the low dose of rbIL-8 reduced ($P = 0.04$) the incidence of HYK compared with controls (Table 2.3). Additionally, a contrast used to assess the overall effect of therapy (L-IL8 and H-IL8 vs. CTR) on the incidence of HYK, indicated that rbIL-8 treatment significantly decreased ($P = 0.02$) the incidence of HYK when compared with CTR cows (Table 2.3). The incidence of CK was not affected by treatment (CTR = 6.0, L-IL8 = 3.7, H-IL8 = 0.0%; $P = 0.15$).

Concentration of IL-8, fatty acids, BHB, and haptoglobin (study 1)

Serum levels of IL8 was significantly increased ($P = 0.04$) for H-IL8 cows when compared to CTR cows (Figure 2.2). However, we detected that both L-IL8 and H-IL8 groups had higher ($P < 0.05$) IL-8 serum concentration at d 3 compared with CTR group (Figure 2.2).

A treatment by parity interaction was observed for fatty acids serum concentrations ($P < 0.001$). Multiple comparison tests depicted that primiparous cows treated with H-IL8 had lower ($P = 0.008$) concentrations of fatty acids in serum compared with CTR (Figure 2.3A). Conversely, multiparous cows treated with H-IL8 had higher ($P = 0.01$) serum fatty acids concentrations than CTR cows (Figure 2.3A). We also observed that multiparous cows treated with rbIL-8 had higher ($P \leq 0.05$) fatty acids levels at d 1 than CTR multiparous cows. Serum concentrations of BHB did not differ ($P = 0.18$) between treatment groups (Figure 2.3C). Finally, haptoglobin concentrations were not different ($P = 0.96$) between treatment groups (Figure 2.3D).

Body condition score and reproductive performance

Study 1. Body condition score loss from calving until 35 d after parturition was not affected ($P = 0.30$) by treatment (Figure 2.4A). Additionally, treatment did not alter ($P = 0.95$) the hazard of

pregnancy during the first 280 DIM (adjusted hazard ratio: L-IL8 = 0.91, 95% CI: 0.57 to 1.47; H-IL8 = 0.98, 95% CI: 0.59 to 1.63).

Study 2. No differences were observed ($P = 0.95$) on body condition score loss from calving until 35 d after parturition among treatment groups (Figure 2.4B).

Lactation Performance

Study 1. The effects of treatment on milk, FCM, and ECM yields during the first 6 months after calving are presented on Table 2.4 and Figure 2.5A. Treated cows produced approximately 3.3 kg/d more milk compared with controls (Table 2.4). Relative to CTR, rbIL-8 cows produced approximately 2.7 kg/d more FCM ($P = 0.004$; Table 2.4). We observed that cows treated with rbIL-8 produced approximately 3.0 kg/d more ECM compared with CTR cows ($P = 0.001$; Table 2.4).

Study 2. The effect of treatment on milk, FCM, and ECM yields during the first 6 months after calving are presented in Table 2.4 and Figure 2.5B. As observed in study 1, treatment increased milk, FCM, and ECM yields ($P < 0.001$). We observed that L-IL8, M-IL8, and H-IL8 cows produced 1.2, 2.2, and 2.4 kg/d more milk when compared with controls, respectively (Table 4). Cows in the L-IL8, M-IL8, and H-IL8 groups produced 2.2, 3.0, and 3.4 kg/d more FCM than CTR cows, respectively (Table 2.4). Moreover, cows in the L-IL8, M-IL8, and H-IL8 groups produced 1.8, 2.8, and 3.2 kg/d more ECM when compared with the CTR group, respectively (Table 2.4).

Table 2.1. Descriptive data for cows enrolled in studies 1 and 2. One-way ANOVA and chi-square analysis were used for comparing continuous and categorical data, respectively.

	Study 1				*Study 2*				
Item	L-IL8	H-IL8	CTR	*P*-value[1]	L-IL8	M-IL8	H-LI8	CTR	*P*-value[1]
Median parity	1.5	1.5	1.5	–	–	–	–	–	–
No. of primiparous, n	41	31	32	–	41	41	41	41	–
No. of multiparous, n	39	35	35	–	–	–	–	–	–
Total enrolled animals, n	80	66	67	–	41	41	41	41	–
Average BCS[2] at enrollment	3.41	3.40	3.36	0.42	3.59	3.59	3.67	3.62	0.60
Average RT[3] at enrollment, °C	38.8	38.8	38.7	0.60	38.9	38.9	38.9	39.0	0.52
Average days carried calf, d	277.4	275.2	274.9	0.17	278.0	277.6	280.0	277.4	0.13
Twins, %	0.00	3.00	5.00	0.08	0.00	2.44	0.00	0.00	0.98
Dystocia, %	3.75	1.50	3.00	0.87	4.88	9.76	4.88	4.88	0.84
Stillbirth, %	6.25	3.03	4.48	0.66	4.88	12.2	7.32	4.88	0.68
Male calf, %	46.0	38.0	34.0	0.31	36.6	34.1	46.3	34.1	0.62

Study 1: CTR = 250 mL of saline solution; L-IL8 = 11.25 µg of rbIL-8 diluted in 250 mL of saline solution; H-IL8 = 1,125 µg of rbIL-8 diluted in 250 mL of saline solution.

Study 2: CTR = 250 mL of saline solution; L-IL8 = 0.14 µg of rbIL-8 diluted in 250 mL of saline solution; M-IL8 = 14 µg of rbIL-8 diluted in 250 mL of saline solution; H-IL8 = 1,400 µg of rbIL-8 diluted in 250 mL of saline solution.

[1]For the chi-square test, we followed the assumption that no cell should have an expected frequency of less than 5; when the assumption was not satisfied, Fisher's exact test was used.

[2]BCS = Body condition score.

[3]RT = Rectal temperature.

Table 2.2. Effects of different rbIL-8 doses on the incidence of puerperal metritis and endometritis in cows from study 1.

Group	% Incidence (n/n) Puerperal metritis	Odds ratio (95% CI)	*P*-value Trt[1]	Trt × parity	D[2]
Primiparous					
CTR	12.5 (4/32)	Reference			
L-IL8	26.8 (11/41)	2.71 (0.59-12.4)			0.26
H-IL8	32.3 (10/31)	4.65 (0.94-22.8)	0.63	0.02	0.11
Multiparous					
CTR	34.3 (12/35)	Reference			
L-IL8	8.11 (5/39)	0.13 (0.02-0.70)			0.03
H-IL8	6.35 (5/35)	0.18 (0.04-0.91)			0.03
	Endometritis				
CTR	15.25 (9/59)	Reference			
L-IL8	12.68 (9/71)	0.43 (0.01-1.89)	0.32	0.68	-
H-IL8	12.28 (7/57)	0.33 (0.06-1.80)			

Study 1: CTR = 250 mL of saline solution, n = 67; L-IL8 = 11.25 µg of rbIL-8 diluted in 250 mL of saline solution, n = 80; H-IL8 = 1,125 µg of rbIL-8 diluted in 250 mL of saline solution, n = 66.
[1]Trt, treatment.
[2]D = different rbIL-8 treatment groups were tested against the control group using Dunnett's procedure.

Table 2.3. Effects of different rbIL-8 doses on the incidence of hyperketonemia in cows from study 1.

	% Incidence (n/n)	Odds ratio	*P*-value[1]		
Group	Hyperketonemia[2]	(95% CI)	Trt	D	C
CTR	30 (6/20)	Reference		–	
L-IL8	15 (3/20)	0.37 (0.16-0.85)	0.05	0.04	0.02
H-IL8	20 (4/20)	0.51 (0.23-1.11)		0.16	

Study 1: CTR = 250 mL of saline solution, n = 67; L-IL8 = 11.25 µg of rbIL-8 diluted in 250 mL of saline solution, n = 80; H-IL8 = 1,125 µg of rbIL-8 diluted in 250 mL of saline solution, n = 66.
[1]Trt, treatment; C = contrast between CTR and all rbIL-8 doses combined; D = different rbIL-8 treatment groups were tested against the control group using Dunnett's procedure.
[2]The incidence of hyperketonemia was evaluated from the subset cows from which blood was collected (20/treatment).

Table 2.4. Effect of different rbIL-8 doses on production parameters of cows from studies 1 and 2 during the first six months after calving.

Study	Milk (LSM±SEM)	*P*-value[1]	FCM (LSM±SEM)	*P*-value[1]	FCM (LSM±SEM)	*P*-value[1]
			Kg/d			
Study 1						
CTR	31.5±1.2		35.0±1.2		33.5±1.2	
L-IL8	34.8±1.3	<0.001	37.6±1.6	0.004	36.4±1.3	0.001
H-IL8	34.8±1.3		37.7±1.6		36.6±1.3	
Study 2						
CTR	31.7±1.8		32.5±1.9		32.6±1.9	
L-IL8	32.9±1.8	<0.001	34.7±1.9	<0.001	34.4±1.9	<0.001
M-IL8	33.9±1.7		35.5±1.8		35.4±1.8	
H-IL8	34.1±1.7		35.9±1.8		35.9±1.8	

Study 1: CTR = 250 mL of saline solution, n = 67; L-IL8 = 11.25 µg of rbIL-8 diluted in 250 mL of saline solution, n = 80; H-IL8 = 1,125 µg of rbIL-8 diluted in 250 mL of saline solution, n = 66.

Study 2: CTR = 250 mL of saline solution, n = 41; L-IL8 = 0.14 µg of rbIL-8 diluted in 250 mL of saline solution, n = 41; M-IL8 = 14 µg of rbIL-8 diluted in 250 mL of saline solution, n = 41; H-IL8 = 1,400 µg of rbIL-8 diluted in 250 mL of saline solution, n = 41.

[1]*P*-values indicate overall treatment effects.

Figure 2.1. Rectal temperature (°C) of cows treated with different rbIL-8 doses and controls from study 1. Postpartum cows (n = 213) were randomly allocated into one of three intrauterine treatment groups: control (CTR; 250 mL of saline solution), low-dose (L-IL8; 11.25 µg of rbIL-8 diluted in 250 mL of saline solution), and high-dose (H-IL8, 1,125 µg of rbIL-8 diluted in 250 mL of saline solution). Results are presented at LSM ± SEM. * $P \leq 0.05$.

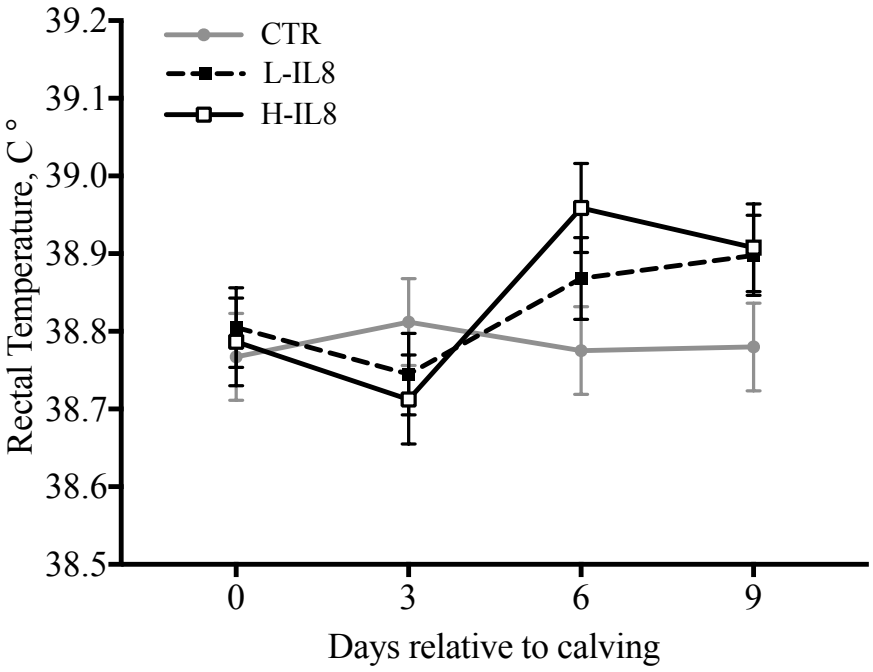


Figure 2.2. Interleukin-8 (IL-8) plasma concentrations of cows treated with different rbIL-8 doses and controls from study 1. Postpartum cows (n = 213) were randomly allocated into one of three intrauterine treatment groups: control (CTR; 250 mL of saline solution), low-dose (L-IL8; 11.25 µg of rbIL-8 diluted in 250 mL of saline solution), and high-dose (H-IL8, 1,125 µg of rbIL-8 diluted in 250 mL of saline solution). Blood samples were harvested from a subset of cows (20/treatment group). Results are presented at LSM ± SEM. * $P \leq 0.05$.

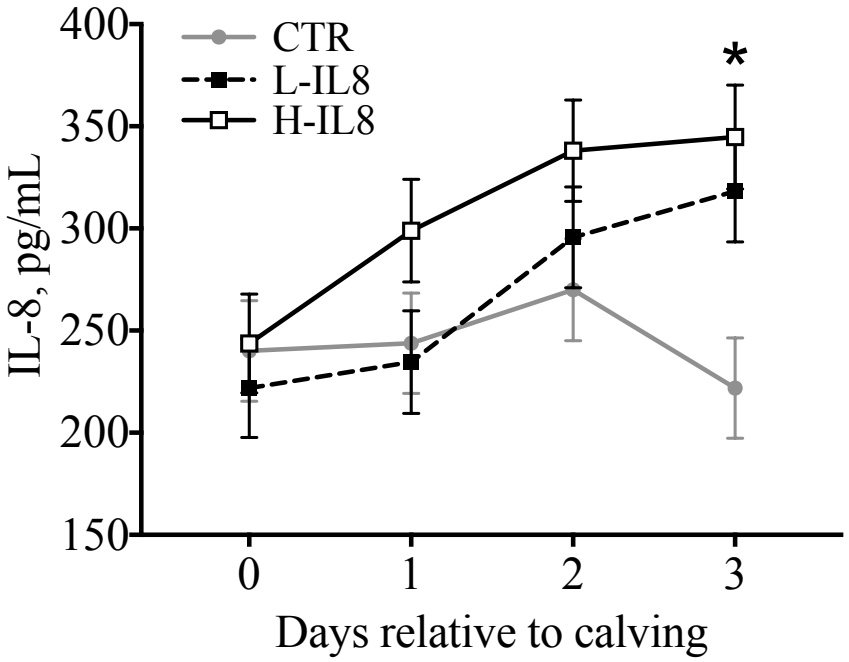


Figure 2.3. Fatty acids serum levels of primiparous (**A**) and multiparous (**B**) cows, and β-hydroxybutyrate (BHB; **C**), and haptoglobin (**D**) serum levels of cows treated with rbIL-8 and controls from study 1. Postpartum cows (n = 213) were randomly allocated into one of three intrauterine treatment groups: control (CTR; 250 mL of saline solution), low-dose (L-IL8; 11.25 µg of rbIL-8 diluted in 250 mL of saline solution), and high-dose (H-IL8, 1,125 µg of rbIL-8 diluted in 250 mL of saline solution). Blood samples were harvested from a subset of cows (20/treatment group). Results are presented at LSM ± SEM. * $P \leq 0.05$.

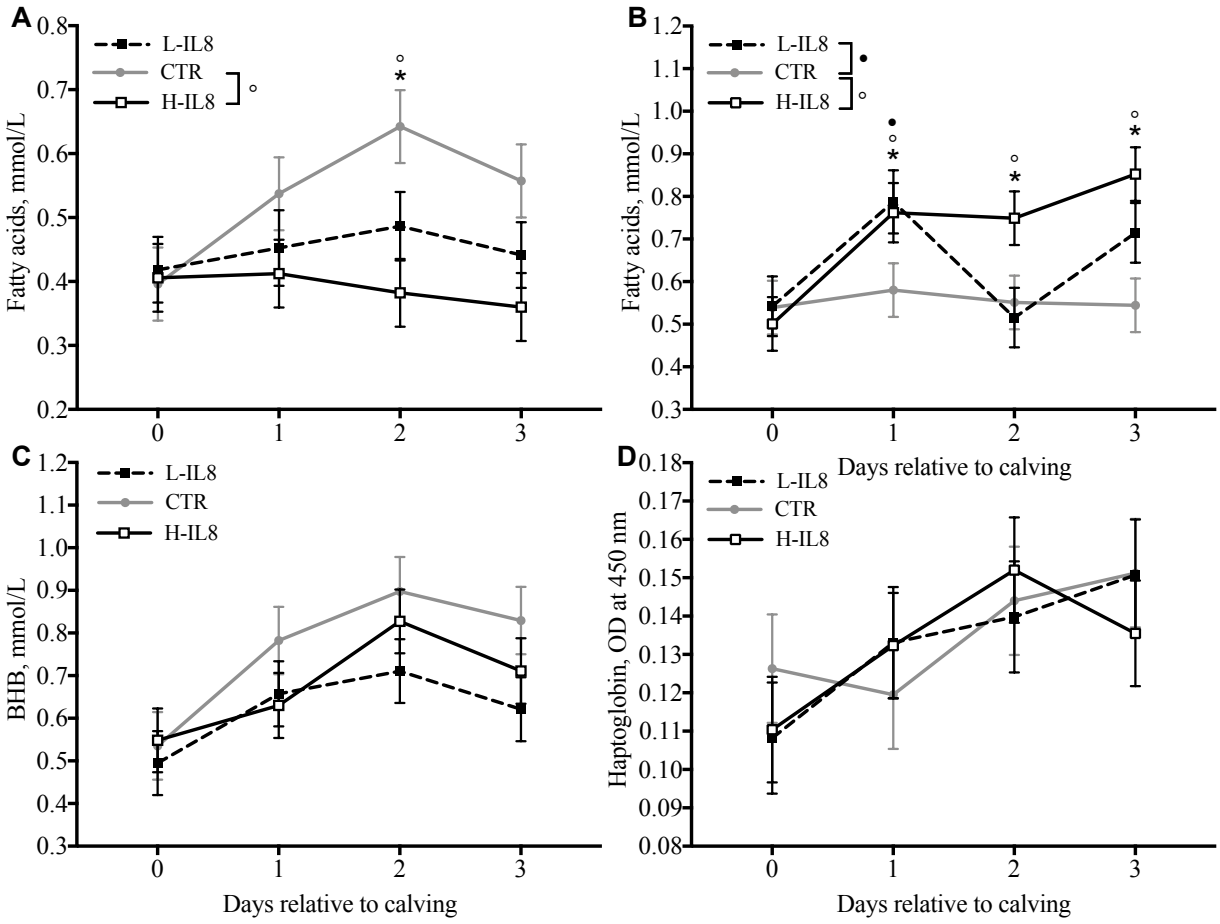


Figure 2.4. Body condition score loss from day of enrollment (day of parturition) until 35 days in milk of cows treated with different doses of rIL8 and controls from studies 1 (**A**) and 2 (**B**). For study 1, 213 cows were randomly allocated into one of three intrauterine treatment groups: control (CTR; 250 mL of saline solution), low-dose (L-IL8; 11.25 µg of rbIL-8 diluted in 250 mL of saline solution), and high-dose (H-IL8, 1,125 µg of rbIL-8 diluted in 250 mL of saline solution). For study 2, 164 cows were randomly allocated into one of four treatment groups: control (CTR, 250 mL of saline solution), low-dose (L-IL8, 0.14 µg of rbIL-8), medium-dose (M-IL8, 14 µg of rbIL-8), and high-dose (H-IL8, 1,400 µg of rbIL-8). Results are presented at LSM ± SEM.

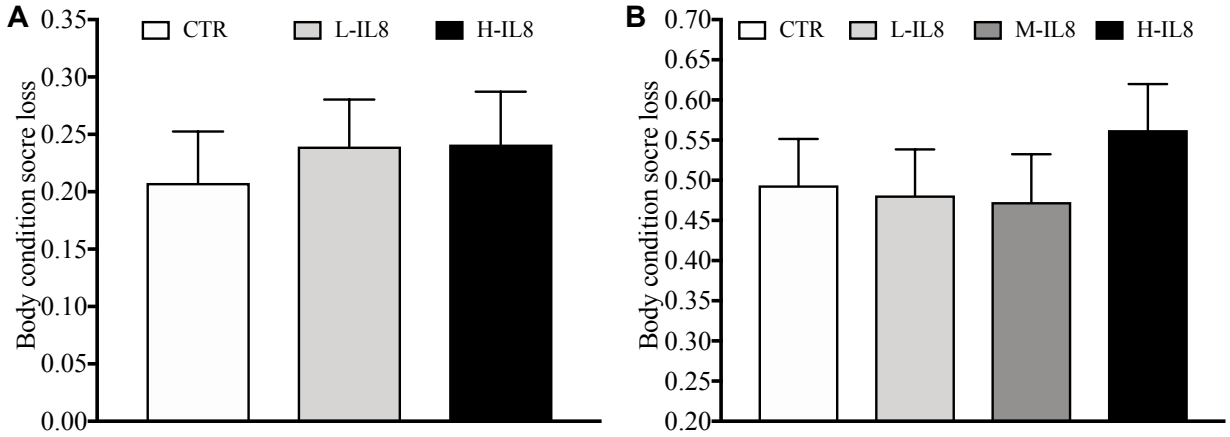
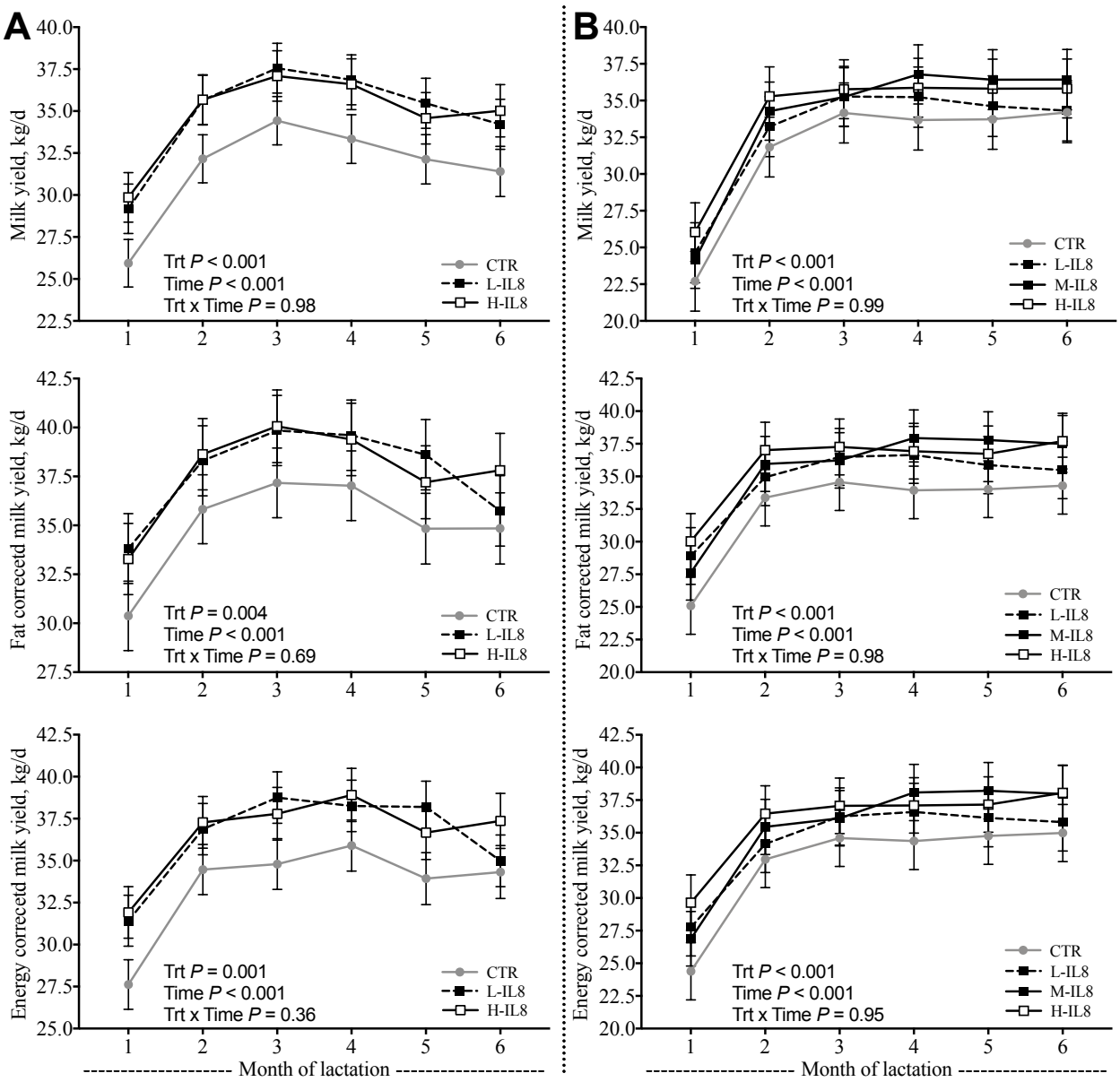


Figure 2.5. Monthly milk yields (kg/d), 3.5% fat corrected milk yields (kg/d), and energy corrected milk yields (kg/d) after parturition of cows treated with different doses of rIL8 and controls from studies 1 (**A**) and 2 (**B**). For study 1, 213 cows were randomly allocated into one of three intrauterine treatment groups: control (CTR; 250 mL of saline solution), low-dose (L-IL8; 11.25 µg of rbIL-8 diluted in 250 mL of saline solution), and high-dose (H-IL8, 1,125 µg of rbIL-8 diluted in 250 mL of saline solution). For study 2, 164 cows were randomly allocated into one of four treatment groups: control (CTR, 250 mL of saline solution), low-dose (L-IL8, 0.14 µg of rbIL-8), medium-dose (M-IL8, 14 µg of rbIL-8), and high-dose (H-IL8, 1,400 µg of rbIL-8). *P*-value for the fixed effect of treatment, time and the interaction term treatment by time are included in each graph. Results are presented at LSM ± SEM.



DISCUSSION

Herein we demonstrated in two independent studies that intrauterine rbIL-8 administration in the immediate postpartum was associated with a significant increase in milk production. In study 1, cows treated with intrauterine rbIL-8 at calving produced approximately 3.3, 2.7, and 3.0 kg/d more milk, FCM, and ECM yields, respectively, compared with CTR cows during the first 6 months after parturition. The observed effect of rbIL-8 on milk yield in study 1 was replicated in study 2; where rbIL-8 cows produced significantly more milk, FCM, and ECM yields when compared with CTR cows. This is the first scientific manuscript that has demonstrated that milk production can be enhanced with the use of rbIL-8. In addition, the administration of rbIL-8 reduced the incidence of HYK and puerperal metritis in multiparous cows.

Interleukin-8 is a chemokine that binds to G protein-coupled receptors (CXCR1 or CXCR2) to activate and promote chemotaxis of neutrophils (Holmes et al., 1991, Mitchell et al., 2003). In the last decades the list of functions attributed to IL-8 is expanding. For instance, due to the presence of a sequence motif (Glu-Leu-Arg), named ELR, IL-8 belongs to a subset of chemokines associated with angiogenic properties (Onuffer and Horuk, 2002). Moreover, recent experiments have demonstrated that IL-8 exerts mitogenic and antiapoptotic effects (Colletti et al., 1998, Hogaboam et al., 1999, Osawa et al., 2002).

Lipolysis is dramatically increased during the postpartum period because of increased responsiveness of adipose tissue to catecholamines, low blood insulin concentration, and decreased peripheral insulin sensitivity (McNamara and Hillers, 1986, De Koster and Opsomer, 2013, Saremi et al., 2014). All these metabolic changes are part of the homeorhetic shifts that postpartum cows experience to support lactation nutrient demands (Bell and Bauman, 1997). Upon binding to its receptor, insulin triggers a cascade of events that involve several proteins, such as insulin receptor substrates (IRS), phosphatidylinositol-3-kinase (PI3K), and 3-phospohoinositide-dependent protein kinase 1 (PDK1), which leads to the activation of AKT protein kinase (Chang et al., 2004). Activation of AKT results in GLUT-4 translocation and

glucose uptake (Kohn et al., 1996). Interleukin-8 is a pro-inflammatory cytokine associated with development of insulin resistance (Fujishiro et al., 2003, Kobashi et al., 2009, Hardy et al., 2011). Kobashi et al. (2009) demonstrated *in vitro* that IL-8 can promote insulin resistance via the inhibition of AKT activation through MAPK/ERK pathway. In addition, stimulation of toll-like receptors of adipocytes with bacterial particles induced the expression of IL-8, impaired insulin-mediated glucose transport, stimulated lipolysis, and led to insulin resistance (Franchini et al., 2010), which are all metabolic changes that assure glucose availability to support the immune response to LPS. Therefore, it is possible that the dramatic increases in milk production herein observed because of rbIL-8 use is a consequence of increased insulin resistance; which could be supporting nutrient partitioning towards the mammary gland.

The increase in milk production observed in dairy cows treated with exogenous bovine somatotropin (**bST**) occurs thought complex and coordinated physiological processes that involve multiple tissues, metabolites, and hormones. One of the major effects of bST is to promote insulin resistance (Bauman and Vernon, 1993). Furthermore, a recent study revealed that feeding mid-lactation cows with palmitic acid promotes insulin resistance through sphingolipid ceramide-dependent mechanisms (Mathews et al., 2016). In that study, treated cows showed increased milk yield even after the palmitic acid was removed from the diet (Mathews et al., 2016). Therefore, facilitating glucose partitioning by decreasing insulin action seems to be a key strategy to enhance milk production.

Growth hormone stimulates the liver to produce insulin-like growth factor-I (**IGF-I**). However, during the early lactation, high blood GH concentration cannot restore blood IGF-1 levels due to the reduced expression and abundance of liver-specific GH receptor (Rhoads et al., 2007). A negative feedback control of GH actions occurs via the increased concentration of IGF-1. It has been shown that GH signaling could be altered via the production of suppressors of cytokine signaling (SOCS) proteins (Rico-Bautista et al., 2006). Interleukin-8 promotes the production of SOCS (Stevenson et al., 2004). Thus, it is possible that IL-8 inhibited the GH signaling via SOCS production, which will result in lower production of IGF-1 and thus

exacerbating the un-coupling of the GH/IGF-1 axis that characterize a postpartum dairy cow during the early lactation. Further studies evaluating plasma IGF-1 and GH concentrations in Holstein cows treated with rbIL-8 during the early lactation are necessary to address this hypothesis.

Optimal hepatic function is critical for proper transition into lactation. The hepatic release of glucose increases from 1.4, to 2.7, and to 3.5 kg/d between -9, 11, and 33 d relative to parturition due to greater uptake of substrates and expression of gluconeogenesis rate-limiting enzymes (Greenfield et al., 2000, Reynolds et al., 2003, Selim et al., 2014). Moreover, the liver extracts about 20% of the circulating fatty acids and is the major organ involved with lipid metabolism during the transition period (Reynolds et al., 2003). Within the hepatocytes, fatty acids can be esterified into triglycerides, completely oxidized to generate ATP, or partially oxidized into ketone bodies that can be used as an energy source by other tissues and as a substrate for milk fat synthesis. Accordingly, hepatic weight and oxygen consumption increase by 23% and 215% during the first month of lactation, respectively (Reynolds et al., 2003, von Soosten et al., 2011). However, 20 to 40% of dairy cows across different levels of milk production, breeds, and management systems develop metabolic disorders associated with lipid metabolism (Drackley et al., 2006, Duffield et al., 2009, McArt et al., 2012). In the present study, intrauterine rbIL-8 treatment reduced the incidence of postpartum HYK. Moreover, the observed positive effect on milk yield was not associated with increased BCS loss. Therefore, rbIL-8 treatment might stimulate appetite and increase feed intake, and therefore it would not affect significantly BCS and would reduce the incidence of HYK. The results on the incidence of HYK were calculated from a small number of cows (20/treatment). Thus, further studies with a larger sample size are needed to evaluate whether differences in HYK during the early postpartum should be expected with the use of rbIL-8.

Studies with human and rat hepatocytes showed that rIL-8 reduced apoptosis and stimulated cell proliferation *in vitro* (Colletti et al., 1998, Osawa et al., 2002). For instance, antibody neutralization of CXC chemokines impaired liver regeneration in rats subjected to 70%

hepatectomy (Colletti et al., 1998). Moreover, mice treated with rIL-8 and ConA-induced liver damage resulted in lower transaminases activities (AST and ALT) and significantly reduced liver damage (Osawa et al., 2002). It is possible that the treatment with rbIL-8 in our study reduced hepatocyte apoptosis and increased cell proliferation, thus increasing the number of cells available to process nutrients and metabolites. As a result, the liver would have been able to oxidize a larger amount of fatty acids before the TCA cycle was inhibited due to excess of ATP/NADH and depression of appetite associated with hepatic oxidation; the upshot would be reduced BHB production and reduced lipid accumulation within hepatocytes. However, further investigation is needed to address this hypothesis.

The activation of NF-κB during mammary gland development is critical for mammary cell differentiation (Brantley et al., 2001, Cao et al., 2001). Prolactin and progesterone are the primary hormones necessary to ensure proper mammary gland development during pregnancy. Interestingly, it has been demonstrated that both hormones induced the expression of receptor activator of NF-κB ligand (RANKL), which is essential to activate NF-κB and up-regulate cycling D1 (Srivastava et al., 2003). Interleukine-8 is also known as an activator of RANKL (Bendre et al., 2003). Therefore, it is also possible that the observed effect of IL-8 administration on milk yield might be, at least in part, due its potential direct effects on the mammary gland tissue development. Furthermore, IL-8 also promotes angiogenesis through the stimulation of a potent vasculogenic and angiogenic factor, named vascular endothelial growth factor (VEGF), by the activation of different signaling pathways (Strieter et al., 1995, Martin et al., 2009, Hou et al., 2014). Along this line, we speculate that IL-8 might also increase mammary gland and liver blood flow, which would result in an increased oxygen and nutrient supply and ultimately promote tissue proliferation in those organs.

Taken together, considering the effects of rbIL-8 treatment that we observed on circulating metabolites, HYK, and on milk production, and the evidence that IL-8 might induce insulin resistance (Kobashi et al., 2009), increase SOCS proteins (Stevenson et al., 2004), promote angiogenesis (Martin et al., 2009), reduces apoptosis and stimulates cell proliferation

(Colletti et al., 1998, Osawa et al., 2002), we hypothesize that a single administration of rbIL-8 at the day of calving might involve one or several biological processes: increases insulin resistance in peripheral tissues, favors the un-coupling of the GH/IGF-1 axis, increases mammary cell proliferation, and enhances hepatic function during lactation; thus, improving metabolic health and milk yield in Holstein cows. However, the precise mechanism underlying the role of rbIL-8 on milk yield in dairy cows, particularly in controlling homeorhesis, remains to be studied.

Uterine diseases are highly correlated with bacterial contamination and immune suppression (Dubuc et al., 2010, Bicalho et al., 2012). Thus, recruitment of neutrophils to the uterine lumen is a key factor for early clearance of bacterial contamination (Kimura et al., 2002, Hammon et al., 2006). Herein, the intrauterine administration of rbIL-8 significantly reduced the incidence of puerperal metritis in multiparous cows. Although, in the present study, we did not evaluate the proportion of neutrophils in the reproductive tract, we have previously demonstrated that Holstein heifers and non-pregnant lactating cows that received vaginal and intrauterine infusion of rbIL-8 had higher proportion of neutrophils in their reproductive tract when compared with controls (Bicalho et al. 2018). Additionally, our previous work also demonstrated its chemoattractant properties *in vitro* (Bicalho et al. 2018). Thus, we suggest that intrauterine administration of rbIL-8 within 12 h of parturition increases neutrophils migration and activation to the reproductive tract, which would result in early clearance of bacterial contamination.

We did not observe an effect of treatment on puerperal metritis for primiparous cows. Antibodies are the major and most important opsonin molecules of the adaptive immune system (Hiemstra and Daha, 1998). As neutrophils encounter a bacterium, opsonins neutralize the bacterium electrostatic charge by coating them with positively charged molecules, facilitating the interaction of the negatively charged neutrophils with the opsonin-coated bacteria. More importantly, antibodies are required to recognize specific bacteria to signal neutrophils to initiate phagocytosis (Hiemstra and Daha, 1998, Rosales et al., 2016). Because primiparous animals have never been exposed to postpartum uterine disease they are very susceptible to reproductive tract infections in the early postpartum period. It is possible that the lack of effect of rbIL-8

treatment on puerperal metritis in primiparous cows was due to absence of specific antibodies against pathogens associated with puerperal metritis. Another possible explanation is that the dose used in the present study was too high for primiparous cows. Although the intrauterine administration of rbIL-8 did not significantly increase the incidence of puerperal metritis in primiparous cows, we observed a numerical increase in a dose dependent manner. Therefore, is possible that an excessive migration and activation of neutrophils triggered by rbIL-8 treatment could cause a more robust inflammatory response and thus more cows exhibiting altered uterine discharge and systemic clinical signs. More studies are needed to evaluate these hypotheses.

Moreover, it has been hypothesized that RFM is caused by an impaired immune function during the peripartum period, with a special emphasis on neutrophil function and migration. Thus, the failure of placental detachment seems to be, at least in part, due to a reduced ability of neutrophils to digest the cotyledon-caruncle attachment after parturition. Interleukin-8 plays a key role in the pathogenesis of RFM. It was previously demonstrated that cows that developed RFM had impaired neutrophil function and lower plasma IL-8 concentrations before and after calving compared to cows that did not develop RFM (Kimura et al., 2002). In addition, IL-8 was reported to induce neutrophil collagenase and elastase expression, which are enzymatic activities involved in fetal membrane detachment and cervical dilation (Kanayama et al., 1988, Rath et al., 1998). However, the results presented here demonstrate that intrauterine administration of rbIL-8 within 12 h after calving has no preventive effect on RFM occurrence. In the present study, we did not have the power to compare the effect of treatment on incidence of uterine diseases of cows treated early or those treated closer to 12 h after calving. It is possible that rbIL-8 could have a beneficial effect on RFM if it was applied earlier, either before parturition or immediately after delivery of the calf. This hypothesis remains to be evaluated.

CONCLUSIONS

In summary, intrauterine administration of rbIL-8 within 12 h after parturition failed to prevent RFM. However, more research is needed to evaluate immediate administration of rbIL-8

after delivery of the calf on RFM. Intrauterine rbIL-8 treatment reduced the incidence of puerperal metritis in multiparous cows. More importantly, treatment with intrauterine rbIL-8 within 12 h after calving increased milk, FCM, and ECM yields in the long-term. In addition, rbIL-8 administration decreased the incidence of HYK. Further research on lactating cows is needed to evaluate rbIL-8 effects on liver function, dry mater intake, cell proliferation and apoptosis in liver and mammary gland, metabolic parameters, and its potential effect on the homeorhetic shifts that occur after calving to support lactation.

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CHAPTER 3: Effects of recombinant bovine interleukin-8 (rbIL-8) treatment on health, metabolism, and milk production in Holstein cattle III: Systemic administration of rbIL-8 induces whole-body insulin resistance in bull calves

ABSTRACT

This study was conducted to assess the effects of systemic administration of recombinant bovine interleukin-8 (rbIL-8) on response to a glucose challenge, blood metabolites, immune cell populations, and inflammatory parameters in bull Holstein calves. Calves from 30 ± 6 d of life were individually housed and randomly allocated to one of two treatment groups: rbIL-8 (rbIL-8, n = 10) and control (CTR, n = 8). Calves assigned to the rbIL-8 group received 1 s.c. injection (d 1, 0900 h) and 6 i.v. injections (d 1 at 1600 h, d 2 and 3 at 0900 h and 1600 h, and d 4 at 0900 h) of rbIL-8 (4 μg/kg BW), whereas the CTR group received 2 mL of sterile saline solution at each time point. Day of enrollment was considered as d 1 and the study duration was 10 d. Insulin concentrations and whole-body glucose disappearance were evaluated by an intravenous glucose tolerance test conducted at 12 h and 7 d following the last rbIL-8 injection. Rectal temperature and blood samples were collected on d 1, 2, 3 and 4 at -30 (before treatment, 0830 h), 30, 60, 120, 240 and 360 min after treatment, and daily at 0830 h for the rest of the study period. Serum was harvested and the following parameters were measured: β-hydroxybutyrate (**BHB**), non-esterified fatty acids, glucose, insulin, plasma urea nitrogen, haptoglobin, and differential blood count. Calves treated with rbIL-8 had greater concentrations of insulin and area under the curve (AUC) in response to the glucose challenge compared with controls. Administration of rbIL-8 increased rectal temperature (rbIL-8 = 39.3 ± 0.1; CTR = 38.9 ± 0.1 °C), BHB concentrations (rbIL-8 = 3.54 ± 0.10; CTR = 2.99 ± 0.12 mg/dL), counts of lymphocytes (rbIL-8 = 4.52 ± 0.12; CTR = 3.84 ± 0.14 × 10^3/μL), monocytes (rbIL-8 = 0.87 ± 0.03; CTR = 0.67 ± 0.04 × 10^3/μL), and granulocytes (rbIL-8 = 3.54 ± 0.22; CTR = 2.66 ± 0.24 × 10^3/μL). We conclude that rbIL-8 induces long-lasting insulin resistance in bull Holstein calves, accompanied by systemic inflammation, and altered blood metabolites, and white blood cell populations.

Keywords: Insulin resistance, interleukine-8, calves

INTRODUCTION

Recently, we demonstrated that a single intrauterine administration of recombinant bovine interleukin-8 (**rbIL-8**) into Holstein cows on the day of parturition is associated with a long-lasting increase in milk production (Zinicola et al., 2018). In that study, intrauterine treatment altered interleukin-8 (**IL-8**) plasma levels, suggesting that the increase in milk yield was caused by a systemic effect of rbIL-8. Additionally, 24 h after treatment, we noted a sharp increase in the concentration of serum fatty acids in cows treated with rbIL-8 (Zinicola et al., 2018). It is well documented in the literature that insulin has both anti-lipolytic and lipogenic effects, resulting in reduced fatty acids mobilization (Herdt, 2000, De Koster and Opsomer, 2013). Therefore, assuming that plasma insulin concentration was similar between treatment groups, the increase in fatty acids levels that we observed could reflect a state of insulin resistance.

High-producing dairy cows undergo extreme metabolic adaptations during the transition from late gestation to early lactation. Feed consumption increases approximately 2-fold between the week preceding parturition and the first 30 d postpartum but remains insufficient to meet lactational nutrient demands (Bell, 1995, Reynolds et al., 2003). During this period of negative nutrient balance, homeorhetic mechanisms trigger the mobilization of body reserves to support milk synthesis. Transient insulin resistance allows glucose to be spared by peripheral tissues and directed toward the synthesis of lactose in the mammary gland (Bell and Bauman, 1997). Furthermore, the reduced insulin response enhances lipolysis and muscle breakdown (De Koster and Opsomer, 2013). As a result of this catabolic state, high-producing dairy cows can lose 1.5 kg/d of body weight in the first three weeks postpartum when energy balance is -7 to -9 Mcal NEL/d (Bell, 1995, Koltes and Spurlock, 2011). The liver is the major organ responsible for processing nutrients and metabolites during the transition period (Reynolds et al., 2003); thus, manipulating homeorhesis while optimizing hepatic function in the early postpartum period is expected to enhance lactational performance. One clear example of an exogenous treatment that alters homeorhetic controls to increase milk production in lactating dairy cows is recombinant

bovine somatotropin (**rbST**). Treatment with rbST increases milk production through complex, coordinated effects in multiple tissues, for example by increasing hepatic gluconeogenesis, inducing insulin resistance, decreasing glucose oxidation, and modulating the activity and function of the mammary gland (Bell and Bauman, 1997). However, repeated administration of rbST is necessary to sustain its positive effect on milk yield, which requires labor and treatment costs.

There are numerous studies suggesting a key role of pro-inflammatory cytokines, such as IL-8, IL-6, and TNF-α, in mediating insulin resistance (Kushibiki et al., 2000; Kobashi et al., 2009 and Hardy et al., 2011). For instance, Holstein steers treated s.c. once daily for 12 d with rbTNF-α displayed decreased peripheral insulin sensitivity (Kushibiki et al., 2000). A direct effect of IL-8 on the insulin response has only been demonstrated *in vitro*, where exposure to IL-8 induced insulin resistance in human adipocytes (Kobashi et al., 2009). Insulin resistance is one of the major homeorhetic regulations that occurs in dairy cows to support lactation. Thus, adjusting energy partitioning by altering tissue responses to insulin has the potential to increase milk production.

Therefore, we hypothesized that systemic administration of rbIL-8 to bull Holstein calves affects response to a glucose challenge, increases systemic inflammation, and alters white blood cell populations. Therefore, the objectives were to explore the effects of rbIL-8 on the insulin response in peripheral tissue, on metabolites, on inflammatory parameters, and on white blood cell counts in bull Holstein calves.

MATERIAL AND METHODS

Ethics Statement

The research protocol was reviewed and approved by the Cornell University Institutional Animal Care and Use Committee (protocol number 2016-0017). The methods were carried out in accordance with the approved guidelines.

Animals, Facilities, and Management

Eighteen Holstein bull calves between 17 and 29 d old and between 39.5 and 73.3 kg of body weight (**BW**) were purchased from a commercial dairy farm and shipped to the research barn situated in the large animal sector of Cornell University, Ithaca, NY. Calves born only by normal calving were included in the study. After calving, calves were fed with 4 L of pooled pasteurized colostrum within 6 h of birth. Each individual pen (concrete walls with stainless steel gate; 2 m^2) was bedded with pine shavings and cleaned on a daily basis. Animals were acclimated for 7 d prior to initiation of the study. Calves were fed with 9 L of raw milk, purchased from the Cornell University Teaching Dairy, twice daily (0630 and 1600 h). Additionally, water was available between feedings, and calf starter (Calf starter 18% CP, DuMOR) was offered *ad libitum*.

Experimental Design and Sample Collection

The day of enrollment was considered as d 1 and the study duration was 10 d. Calves were randomly allocated to one of two treatment groups: rbIL-8 (**rbIL-8**, n = 10) or control (**CTR**, n = 8). Originally the study was designed to administer the treatments s.c. However, following the first treatment, a large local inflammatory reaction was observed, so the route of administration was changed to i.v. Therefore, calves assigned to the rbIL-8 group received 1 s.c. injection (d 1, 0900 h) and 6 i.v. injections (d 1 at 1600 h; d 2 and d 3 at 0900 and 1600 h; and d 4 at 0900 h) of rbIL-8 (4 μg/kg BW). Calves assigned to the CTR group received 2 mL of sterile saline solution (VEDCO Inc., Saint Joseph, MO) according to the above schedule. Recombinant bovine IL-8 was produced and purified according to previously described methods (Bicalho et al., 2017). Long-term 16Ga catheters (MILA INTERNATIONAL INC., Florence, KY) were inserted into the left jugular vein 1 d before the first treatment for collection of blood during the study period and for administration of glucose. The catheters were maintained by flushing with heparinized saline solution. Blood samples were collected on d 1, 2, 3 and 4 prior to treatment; at 30 min prior to treatment (0830 h) and at 30, 60, 120, 240 and 360 min after treatment; and daily

at 0830 h for the rest of the study period via jugular vein catheter into vacutainer K2-EDTA Blood Collection Tubes (BD Vacutainer®, Franking Lakes, NJ) and immediately placed on ice. Within 1 h after blood collection, samples were used for hemogram determination using a Vet hemogram instrument (Heska – Hemature^{tm}, Loveland, CO), and plasma was obtained by centrifugation (2,000 × *g* for 15 min at 4°C) and aliquoted into 2-mL eppendorf tubes and stored at -80°C until analysis. Additionally, rectal temperature (**RT**) was recorded daily and after treatment at the same time as the blood sample collections, as described above. Measurements of BW were taken at birth and on d 0 of the study, at 1400-1500 h.

Evaluation of the Peripheral Response to Insulin and Glucose Disappearance

Whole body insulin action was evaluated using intravenous glucose tolerance test (IVGTT) in all calves enrolled in the study. The tests were performed 7 h after the last treatment (d 4, 1600 h) and on d 10 (0800 h) of the study. Calves were fasted for 10 h prior to each test. Calves were infused with 0.25 g/kg of BW of glucose (dextrose 50%, wt/vol; Phoenix Scientific Inc., St. Joseph, MO) followed by 5 mL of sterile saline solution to flush the catheter. Blood was sampled at -15, 0, 15, 30, 45, 60, 90 and 120 min relative to glucose infusion. Catheters were flushed between sampling points with sterile saline solution containing sodium heparin to avoid clotting. The initial 3 mL of blood drawn from the catheter at every sampling point was discarded before the sample was collected to avoid dilution. Blood was collected into vacutainer K2-EDTA Blood Collection Tubes (BD Vacutainer®, Franking Lakes, NJ). Samples were placed on ice immediately and centrifuged at 2,000 × *g* for 15 min at 4°C within 1 h of collection. Plasma was harvested and stored at -80°C until assayed.

Hormone and Metabolite Assays

Plasma concentrations of fatty acids (NEFA-C® kit; Wako Pure Chemical Industries, Richmond, VA), β-hydroxybutyrate (**BHB**) (Williamson and Mellanby, 1974; Sigma-Aldrich, St. Louis, MO), plasma urea nitrogen (**PUN**) (Sigma-Aldrich, St. Louis, MO), and glucose

(Sigma-Aldrich, St. Louis, MO) were determined by colorimetric methods. Haptoglobin concentration was determined using a colorimetric procedure as previously described (Bicalho et al., 2014). Results of haptoglobin concentrations were reported as optical density readings at 450 nm of wavelength. Insulin concentration was determined using a commercial ELISA kit for bovine insulin (Bovine Insulin ELISA, ALPCO®, Salem, NH). Following the procedures previously described, a double RIA was used for measuring the plasma concentrations of insulin like growth factor-1 (**IGF-1**; Butler et al., 2003, Butler et al., 2004) and growth hormone (**GH**; Plaut et al., 1987, Rosemberg et al., 1989). h-IGF-1 receptor grade (lot# EBB-CO1) from Gropep (Adelaine, Australia) was used for iodination and standards. Primary antibody anti-h-IGF1-rabbit (#AFP4892898) was obtained from Dr. A.F. Parlow at the National Hormone and Pituitary Program and diluted 1:55k for the assay. For the bGH assay, standards and iodinated tracer were made from Upjohn Growth Hormone, Lot#12 code #77-001. NIDDK-anti-oGH-2 (rabbit) AFPC0123080 was added at 1:35k as the primary antibody. For both assays the second antibody used for precipitation was sheep anti-rabbit gamma globulin supplied by Dr. W.R.Butler, Cornell University. Endotoxin levels from purified rbIL-8 was measured by a chromogenic assay (Pierce, Chromogenic Endotoxin Quant Kit, ThermoFisher Scientific,Waltham, MA) following manufacture instructions. Endotoxin levels were < 5 EU/mL (data not shown). A total of 2 mL of purified rbIL-8 was used for treatments. Thus, calves treated with rbIL-8 received a solution containing endotoxin levels under the FDA limit of < 5 EU/kg.

Statistical Methods

All statistical analyses were performed using SAS (version 9.4; SAS/STAT, SAS Institute Inc., Cary, NC). The effects of rbIL-8 on blood cell parameters, RT, and plasma concentration of haptoglobin, metabolites, insulin, GH, and IGF-1 were analyzed using mixed general linear mixed models with the MIXED procedure of SAS version 9.4. Normality and homoscedasticity of residuals were assessed using residual plots. Fixed effects of treatment, time, BW at d 0, age at d 0, and the two-way interaction treatment by time were offered to the

model as independent variables and calf was considered a random effect. Manual backward stepwise elimination of variables was undertaken when $P > 0.10$. The baseline values for each of the variables were included in the model as covariates. Several covariance structures were tested, and that with the lowest Akaike information criterion (**AIC**) was selected. Plasma concentrations of glucose and insulin from IVGTT were used to create response curves. The positive incremental area under the curve (**AUC**) of glucose and insulin was calculated in SAS, correcting for baseline based on the trapezoidal method as previously described (Cardoso et al., 2011). Significant differences were considered when $P \leq 0.05$ or a trend if $0.05 < P \leq 0.10$. For all models, significant differences between time points were tested using Bonferroni adjustment for multiple comparisons. Data are reported as LSM ± SEM unless otherwise stated.

RESULTS

Descriptive Results

No differences ($P > 0.65$) on BW at birth, BW at study-d 0, and age at study-d 0 were detected between treatment groups.

Intravenous glucose tolerance test

Glucose AUC did not differ between treatment groups among all IVGTT (Figure 3.1A-B). On the IVGTT performed at 12 h after treatment, plasma concentration of insulin was greater ($P \leq 0.05$) in the rbIL-8 group at 15 and 30 min after glucose infusion and tended to be greater ($P \leq 0.10$) at 45 min compared to the CTR group (Figure 3.1A). On IVGTT performed on study d 10, rbIL-8 calves had greater ($P \leq 0.05$) plasma concentrations of insulin at 15 and 30 min after glucose infusion compared with their CTR counterparts (Figure 3.1B). Compared to CTR calves, rbIL-8 calves tended to have a greater insulin AUC during IVGTT conducted 12 hours after treatment ($P = 0.06$) and had greater ($P = 0.002$) insulin AUC during IVGTT performed on study d 10 (Figure 3.1A-B).

Hormone and Metabolite concentrations

No differences were observed in the plasma concentration of glucose, insulin, GH, and IGF-1 between treatment groups (Figures 3.2 and 3.3). Treatment with rbIL-8 increased ($P \leq 0.01$) BHB plasma concentrations particularly at study d 3 and 8 (Figure 3.2). No differences were observed in plasma concentrations of fatty acids between the two groups (Figure 3.2).

Figure 3.4 illustrates the dynamics of insulin, glucose, BHB and fatty acids after treatment on d 1 (s.c.) and d 2 (i.v.). Treatment did affect the serum concentrations of glucose ($P \leq 0.10$), insulin ($P \leq 0.05$), BHB ($P \leq 0.05$) and fatty acids ($P \leq 0.01$). Compared to CTR calves, rbIL-8-treated calves had a greater ($P \leq 0.05$) glucose concentration at 30 min after the first treatment on d 2 followed by an acute increase ($P \leq 0.01$) in insulin concentrations at 120 min. Plasma BHB concentration tended to be greater on d 2 at 30 min, and increased ($P \leq 0.05$) from 60 to 120 min after treatment in the rbIL-8 group compared with controls. Finally, greater ($P \leq 0.05$) concentrations of fatty acids were observed at 360 min on d 2 in calves that were treated with rbIL-8 compared with controls.

Although an overall effect of treatment on plasma concentrations of PUN was not observed, an interaction between treatment and time was observed ($P \leq 0.01$). Calves treated with rbIL-8 had greater ($P \leq 0.05$) concentrations of PUN in plasma on d 10 compared with controls (Figure 3.5). Treatment by time interaction was also observed ($P \leq 0.01$) for haptoglobin concentrations, wherein calves treated with rbIL-8 had greater ($P \leq 0.05$) concentrations of haptoglobin in plasma on d 2 and d 3 compared with controls (Figure 3.6).

Hemogram parameters and rectal temperature

Results of daily, multi time-point monitoring of RT, and absolute numbers of white blood cells (**WBC**), lymphocytes (**LYM**), monocytes (**MONO**) and granulocytes (**GRAN**) are depicted in Figures 3.7 and 3.8. Treatment with rbIL-8 affected RT ($P = 0.03$), WBC ($P \leq 0.01$), LYM ($P \leq 0.01$), MONO ($P \leq 0.01$), and GRAN ($P \leq 0.01$). Calves treated with rbIL-8 had increased ($P \leq 0.05$) RT at 60 and 120 min after the first treatment on d 2, and a trend was observed 30 min

after the treatment on d 3. Moreover, RT was greater at d 8 ($P \leq 0.05$) and d 10 ($P \leq 0.05$), and tended to be greater at d 9 ($P \leq 0.10$) in the rbIL8-treated group compared with the CTR group (Figure 3.7). Calves in the rbIL-8 treatment group had ($P \leq 0.01$) and tended to have ($P \leq 0.10$) greater counts of WBC at 240 and 360 min after the first treatment on d 1, and at 30 min before the first treatment on d 2, respectively (Figure 3.8). Compared to controls, rbIL-8 calves tended to have ($P \leq 0.10$) greater LYM counts at 30 min after the first treatment on d 2 and at 30 min before the first treatment on d 4, and a significant increase ($P \leq 0.05$) was observed on d 10 (Figure 3.8). Relative to controls, rbIL-8 calves tended to have ($P \leq 0.10$) and had greater ($P \leq 0.05$) counts of MONO at 240 and 360 min after the first treatment on d 1, respectively. Additionally, a trend ($P \leq 0.10$) was observed at 120 min after the first treatment on d 4, where rbIL-8 calves had greater MONO concentration than controls (Figure 3.8). Finally, greater ($P \leq 0.01$) counts of GRAN was observed at 240 and 360 min after the first treatment on d 1 in the treatment group compared with the controls (Figure 3.8).

Figure 3.1. Glucose and insulin responses to the glucose tolerance test in calves treated with rbIL-8 or controls at 12 h (A) and 7 d (B) after the treatments. White bars and dark gray bars represent the area under the curve [AUC; mg/dL (glucose) and ng/mL (insulin) per 120 min] of control and rbIL-8-treated calves, respectively. * $P \leq 0.05$, † $P \leq 0.1$. Results are presented at LSM ± SEM.

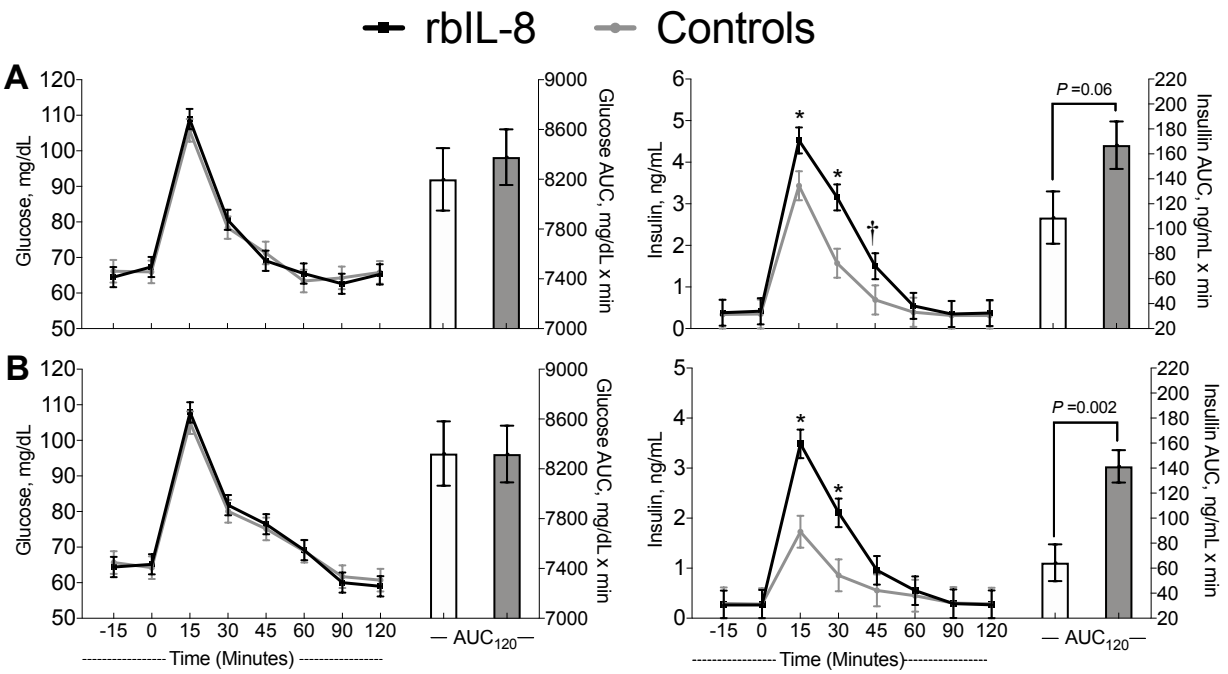


Figure 3.2. Plasma concentrations of glucose, insulin, BHB and fatty acids from d 1 to d 10 of the study of rbIL-8-treated and control calves. The arrows indicate the times of the treatments. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, † $P \leq 0.1$). Results are presented at LSM ± SEM.

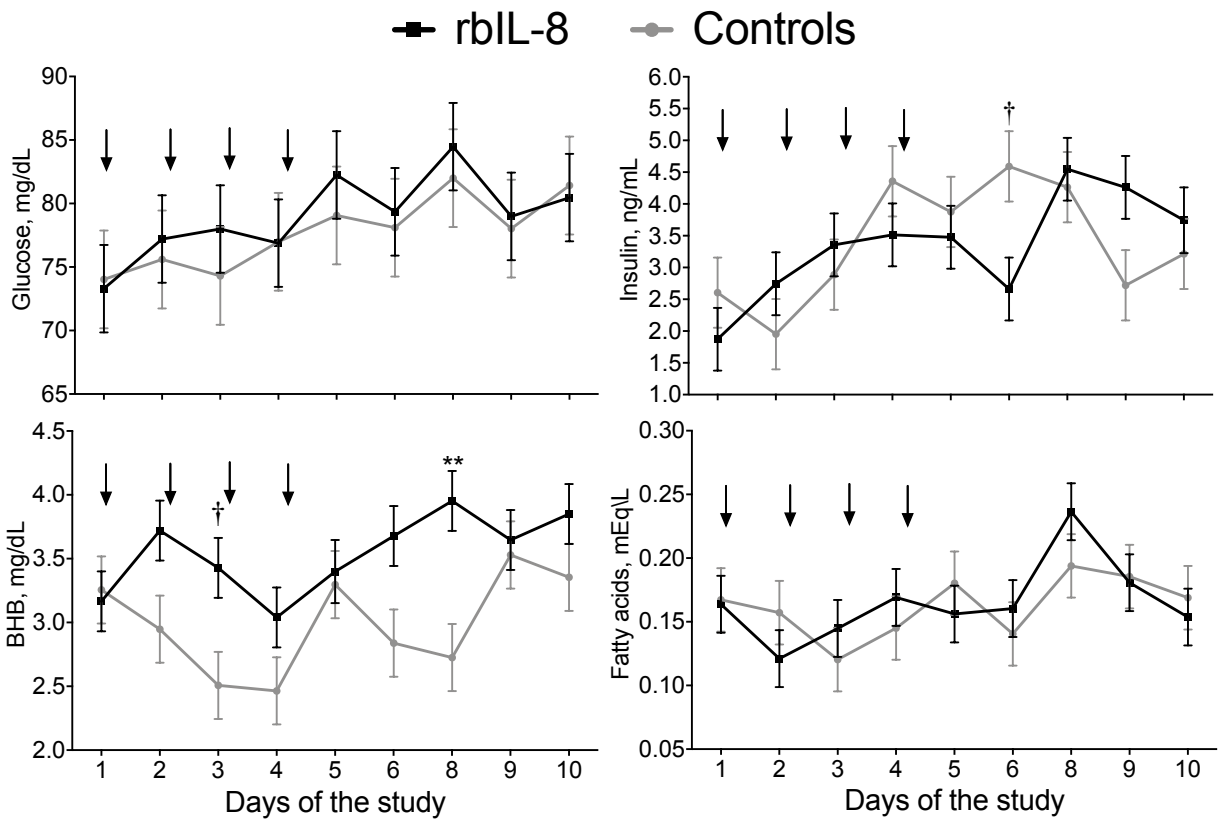


Figure 3.3. Plasma concentrations of GH and IGF-1 at d 1, 5, and 10 of the study of rbIL-8-treated and control calves. Results are presented at LSM ± SEM.

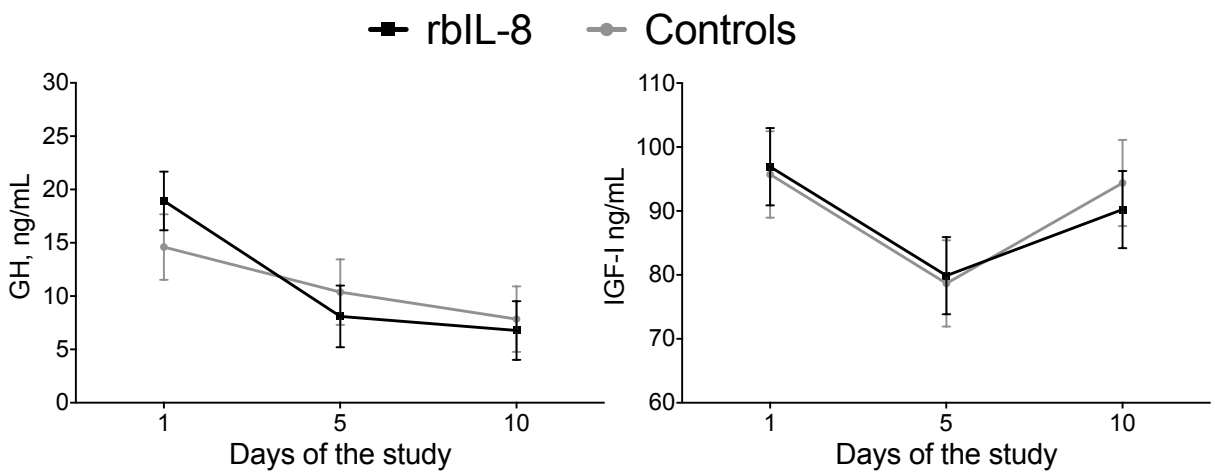


Figure 3.4. Dynamics of the plasma concentrations of glucose, insulin, BHB, and fatty acids after the first treatment (0900 h) on d 1 and 2 of the study of rbIL-8-treated and control calves. The arrows indicate the time of the treatments. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, † $P \leq 0.1$. Results are presented at LSM ± SEM.

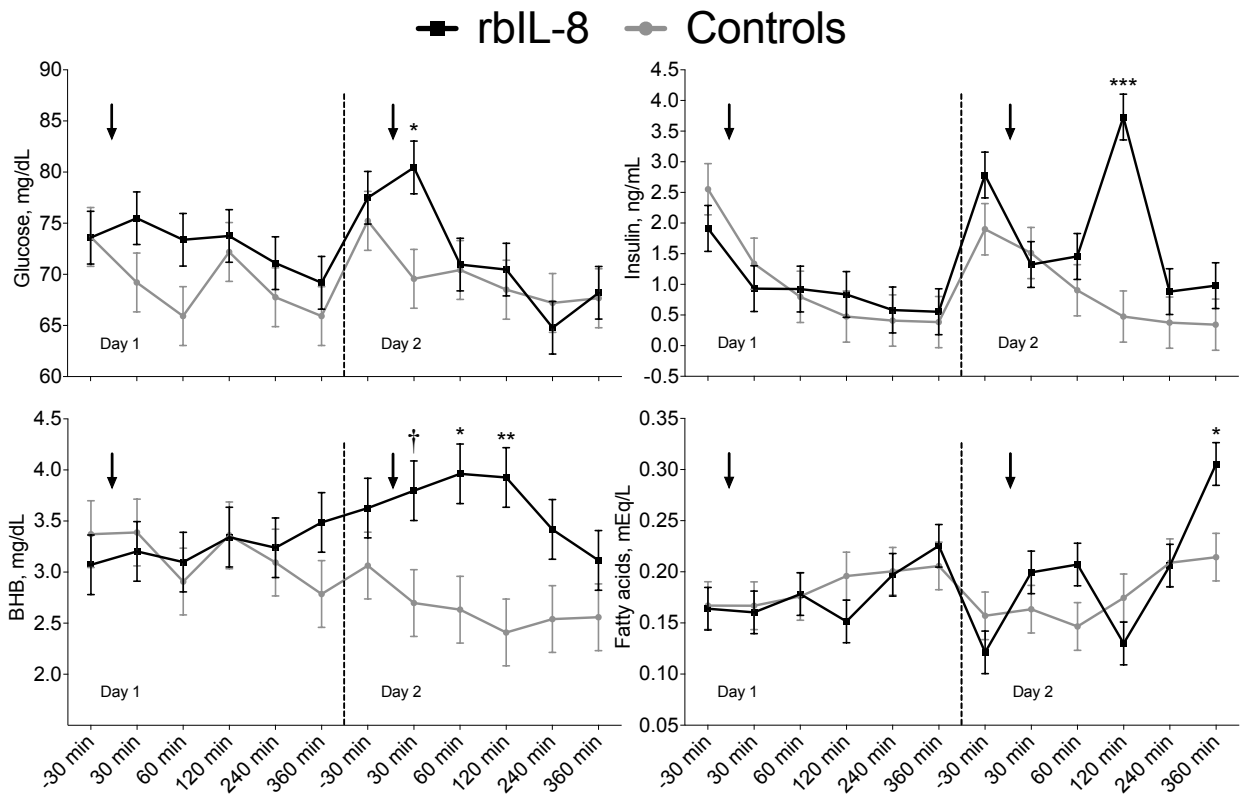


Figure 3.5. Plasma urea nitrogen (PUN) concentration of rbIL-8-treated and control calves from d 1 to d 10 of the study. The arrows indicate the times of the treatments.
*** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, † $P \leq 0.1$. Results are presented at LSM ± SEM.

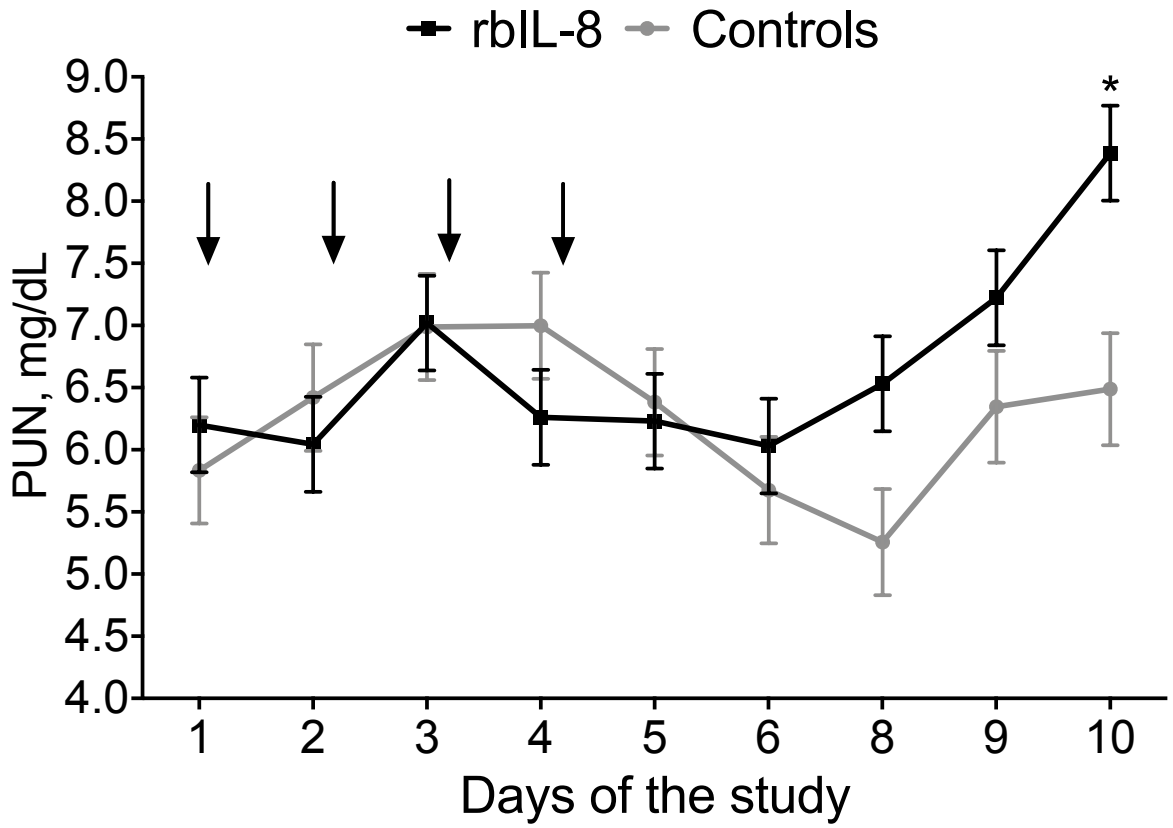


Figure 3.6. Plasma concentration of haptoglobin (optical density units) of rbIL-8-treated and control calves from d 1 to d 10 of the study. The arrows indicate the times of the treatments. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, † $P \leq 0.1$. Results are presented at LSM ± SEM. OD = optical density.

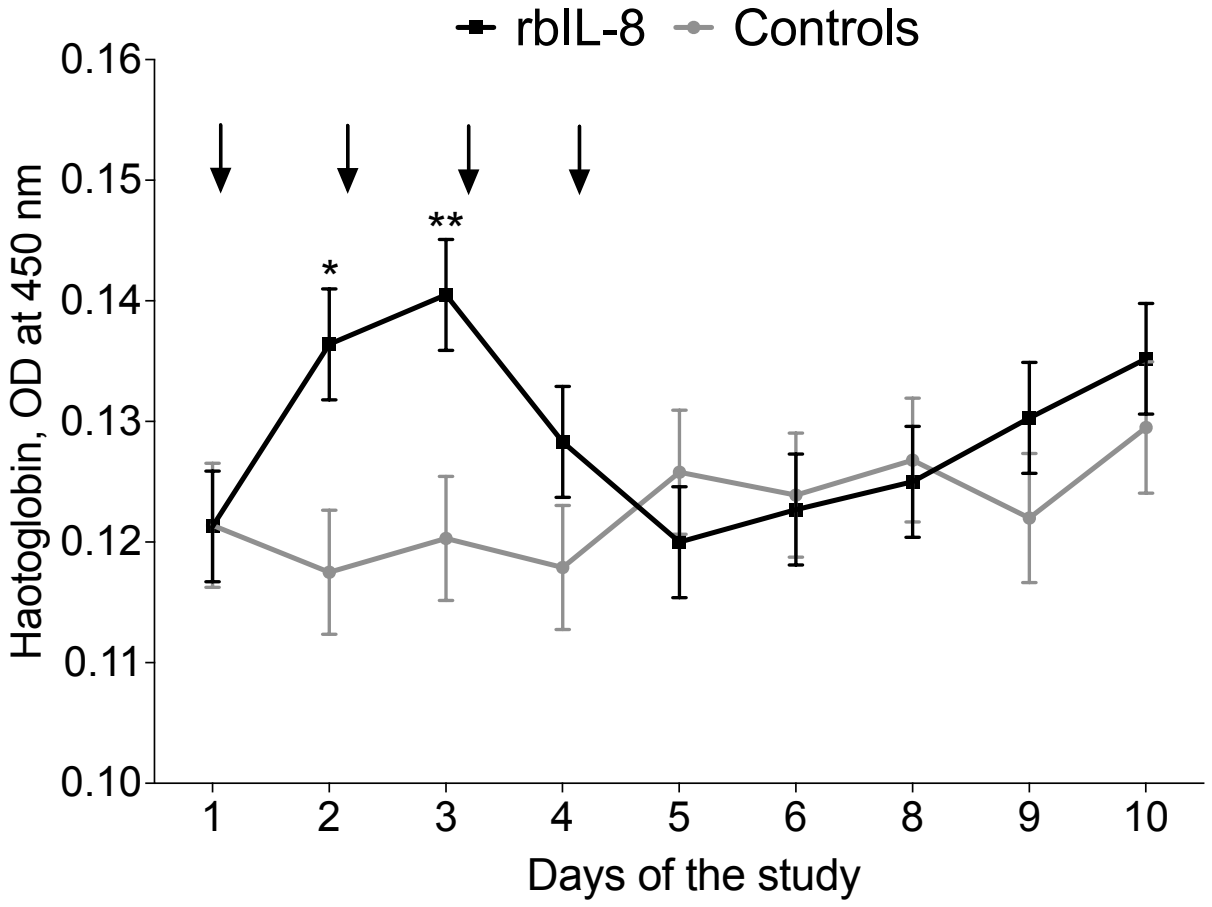


Figure 3.7. Dynamics of rectal temperature following the first treatment (0900 h) on d 1, 2, 3, and 4 of the study of rbIL-8-treated and control calves. The arrows indicate the times of the treatments. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, † $P \leq 0.1$. Results are presented at LSM ± SEM.

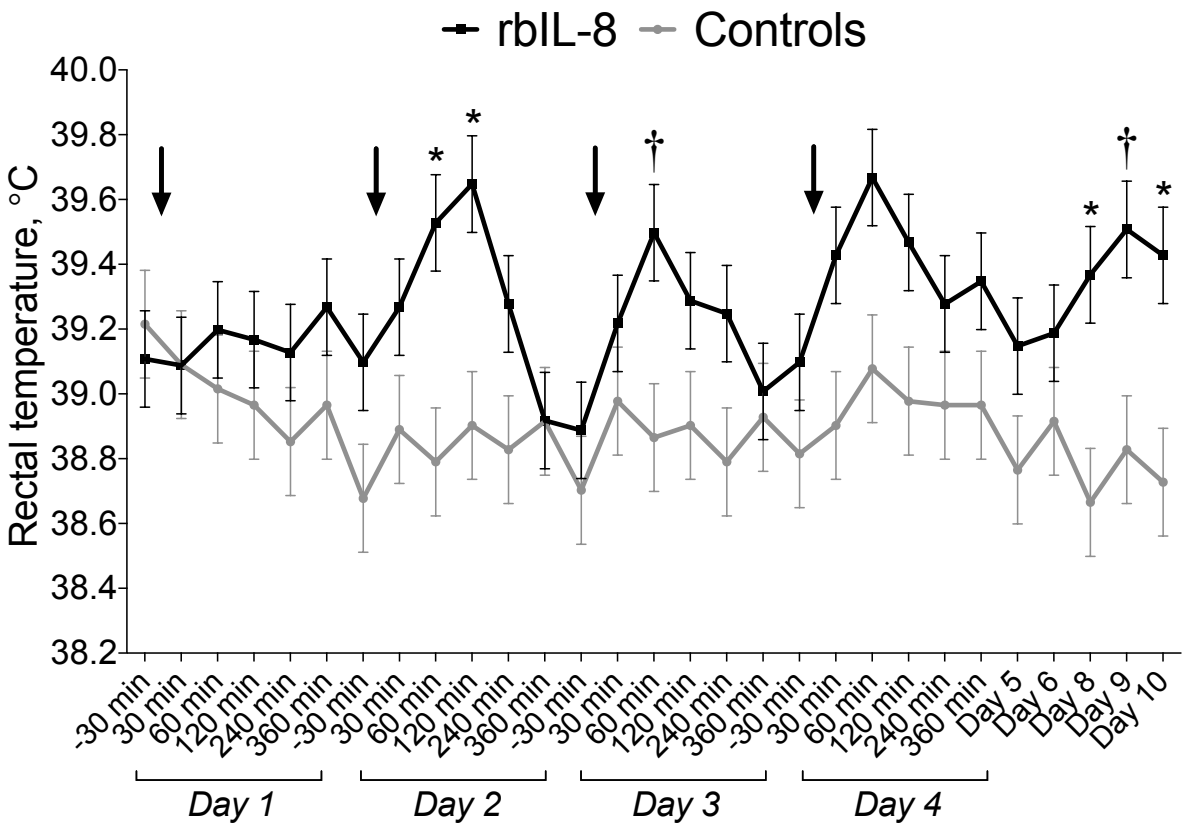
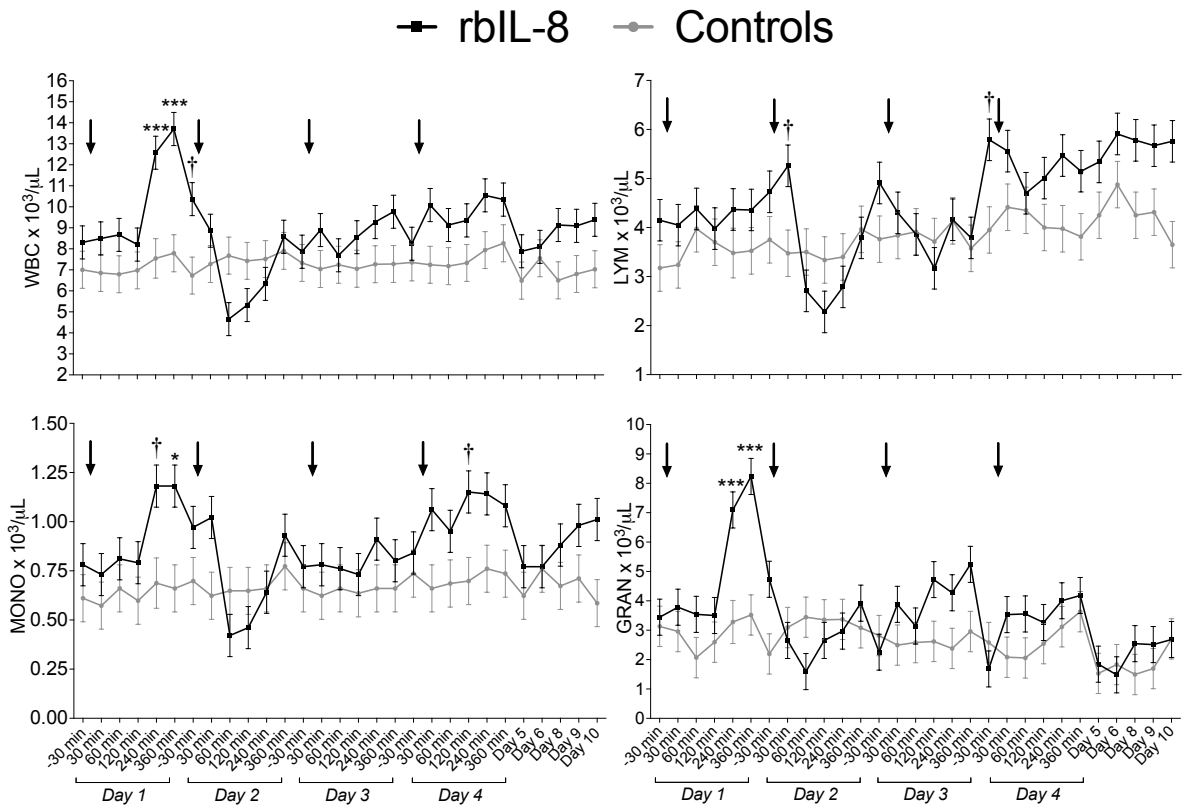


Figure 3.8. Dynamics of the concentration in absolute numbers of white blood cells (WBC), lymphocytes (LYM), monocytes (MONO), and granulocytes (GRAN) following the first treatment (0900 h) on d 1, 2, 3 and 4 of the study of rbIL-8-treated and control calves. The arrows indicate the times of the treatments. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, † $P \leq 0.1$. Results are presented at LSM ± SEM.



DISCUSION

Based on our previous findings of an effect of rbIL-8 on milk production (Zinicola et. al., 2018), the present study was performed to investigate one of the potential underlying physiological mechanisms through which rbIL-8 could enhance lactation performance (Zinicola et. al., 2018). In accordance with our hypothesis, systemic treatment with rbIL-8 induced long-lasting peripheral insulin resistance in bull Holstein calves. In addition, rbIL-8 administration resulted in elevated RT, increased haptoglobin, and altered blood metabolites and white blood cell counts.

In dairy cows, the onset of lactation is supported by dramatic metabolic adaptations that involve key metabolites, hormones and body tissues. Such coordinated metabolic changes that support a physiological state (e.g. lactation) are defined as homeorhesis (Bell and Bauman, 1997). Insulin resistance is described as a reduction in the biological response to insulin in peripheral tissues (De Koster and Opsomer, 2013) and is considered one of the major homeorhetic adaptations that postpartum cows experience to support milk and energy demands (Bell and Bauman, 1997; De Koster and Opsomer, 2013). Interestingly, it has been demonstrated that peripheral tissue insulin responses differ between beef and dairy cattle (Bossaert et al., 2009). In that study, Holstein-Friesian calves (dairy calves) were found to have lower insulin sensitivity compared with beef-breed calves, suggesting that the reduction in insulin sensitivity in the former may be a consequence of genetic selection for milk yield to support lactation (Bossaert et al., 2009).

In an accompanying article, we demonstrated that a single intrauterine administration shortly after parturition of rbIL-8 increased milk yield in a sustained manner in lactating Holstein cows (Zinicola et al., 2018). Although the cellular mechanism underlying the decrease in insulin response in early lactation is not clear, in that study, our data suggested that rbIL-8 might induce insulin resistance. In the present study, a metabolic test (IVGTT) showed that systemic treatments with rbIL-8 induced long-lasting insulin resistance in bull Holstein calves. Additionally, we showed that after the first treatment on d 2, rbIL-8 induced hyperglycemia that

was reflected by an increase in serum insulin. In non-insulin resistant animals, insulin has anti-lipolytic and lipogenic effects. However, instead of identifying a depletion in fatty acids, we observed that rbIL-8-treated calves had a higher serum concentration of fatty acids (coincident with insulin elevation) compared with controls.

In mammals, the role of pro-inflammatory cytokines such as IL-8, IL-6, and TNF-α in altering the insulin response has been studied (Kushibiki et al., 2000, Kobashi et al., 2009, Hardy et al., 2011). For instance, Holstein steers treated once daily for 12 d with rbTNF-α exhibited decreased peripheral insulin sensitivity when assessed with IVGTT and an intravenous insulin tolerance test (Kushibiki et al., 2000). Although, to our knowledge, a direct effect of IL-8 on insulin action in dairy cattle has not been investigated, the results presented here for rbIL-8 on the development of insulin resistance are in accordance with the human literature. The expression of IL-8 in omental fat depots is 2.7-fold greater in obese humans that are insulin-resistant compared with obese patients classified as insulin-sensitive (Hardy et al., 2011). In support, the direct effect of IL-8 on the response to insulin was demonstrated *in vitro*, wherein exposure to IL-8 induced insulin resistance in human adipocytes by reducing the effect of the insulin-stimulated AKT pathway (Kobashi et al., 2009).

Insulin-receptor signaling has a direct effect on activation of the AKT pathway, which promotes GLUT4 translocation to the cell membrane for glucose uptake, stimulates glycogen synthesis, and inhibits gluconeogenesis (Brockman, 1985, Stephens and Pilch, 1995, Barthel and Schmoll, 2003). In addition, the AKT pathway plays a central role in muscle hypertrophy and atrophy (Sandri, 2008). Its activation decreases muscle proteolysis by inhibiting the ubiquitin-proteasome pathway, which results in decreased muscle mobilization (Mann et al., 2016). Although PUN can be influenced by a variety of parameters (e.g., dehydration, dietary protein and energy intake, rumen degradability, and liver and kidney function), it is used as a blood marker to directly reflect the protein status of an animal. For instance, in fasted steers, increased muscle protein turnover to meet energy needs is associated with higher levels of PUN (Ward et al., 1992). Here, although an overall treatment effect on plasma PUN was not observed, a

treatment by time interaction was observed, since on d 10, rbIL-8-treated calves had a higher concentration of PUN compared to controls.

Considering our clear findings for rbIL-8 on altered insulin action, concomitant with the observed increased serum concentrations of BHB and PUN, we suggest that the reduced insulin response led to diminished AKT-phosphorylation (reducing its activity), which could promote the release of glucogenic and ketogenic amino acids from skeletal muscle to the blood stream. Thus, the elevated plasma BHB and PUN levels observed in this study could be due to a systemic rise in amino acid levels owing to increased muscle breakdown. This hypothesis needs to be further investigated.

In the present study, the repeated administration of rbIL-8 caused several changes to the white blood cell population. Interleukin-8 is the main chemoattractant for neutrophils and can be produced by smooth muscle, epithelial cells, endothelial cells, and any cell of the innate immune system with toll-like receptors (Mitchell et al., 2003). In addition, cytokines are well-known promoters of white blood cell differentiation. Here, although rbIL-8 administration altered the blood GRAN concentration, a sustained alteration of GRAN was not observed. Nevertheless, we did detect a chronic shift in white blood cell counts (e.g., for LYM and MONO), a pathology previously linked with obesity-related insulin resistance in humans (Vozarova et al., 2002a,b; Harford et al., 2011). It has been demonstrated that infiltration of macrophages and T cells into peripheral tissues contributes to inflammation and alters insulin action there (Vozarova et al., 2002a, Kintscher et al., 2008, Harford et al., 2011). In addition, it has been shown that a high white blood cell count is associated with a decrease in insulin action and predicts the development of type 2 diabetes (Vozarova et al., 2002b). Thus, it could be hypothesized that rbIL-8 treatments induced activation of the immune system, which was reflected in the altered and elevated white blood cell population. As a result, immune cells might accumulate in peripheral tissues, diminishing the insulin response. Based on the effects rbIL-8 had on the blood immune cells in the present study, we hypothesize that the link between immunity and metabolism could explain the chronic alteration of the insulin response that we observed.

However, further work needs to be completed using specific techniques to detect target immune cells in blood and peripheral tissues before and after rbIL-8 therapy to elucidate the aforementioned hypothesis.

A marker of inflammation evaluated in the present study was the plasma concentration of a member of acute-phase proteins. Haptoglobin is produced by the liver in response to a systemic increase in pro-inflammatory cytokines (Baumann et al., 1989). Here, we demonstrated that repeated administration of rbIL-8 into bull calves induced a significant increase in plasma haptoglobin during the treatment period. Therefore, considering together the detected increases of haptoglobin, RT, and blood cell counts in calves treated with rbIL-8, we suggest that rbIL-8 elicited those effects by acting as an inflammatory stimulus.

Lastly, we explored the effect of rbIL-8 treatment on plasma GH and IGF-1 concentrations. During the early lactation, the GH/IGF-1 axis is uncoupled. This uncoupling favors nutrient partitioning, and therefore high milk production (Rhoads et al., 2007, Lucy et al., 2009). Cytokines are known to inhibit the GH signaling via production of suppressors of cytokine signaling (SOCS) proteins (Rico-Bautista et al., 2006). Thus, we suggested that the observed higher milk yield in cows treated with rbIL-8 might be as a result of IL-8 favoring the un-coupling of the GH/IGF-1 axis (Zinicola et al., 2018). In contrast with our hypothesis, in the present study GH and IGF-1 levels were not affected by rbIL-8 treatment. However, the potential effect of rbIL-8 treatment on GH and IGF-1 concentrations in Holstein cows during the early lactation remains further investigations.

In the present study, we demonstrated that rbIL-8 administration alters insulin sensitivity, which potentially involves rbIL-8 exerting an effect at an immunological–metabolic interface. Based on our previous finding of rbIL-8 enhancing milk yield in lactating cows (Zinicola et al., 2018) combined with the results presented in this study, our current hypothesis is that rbIL-8 promotes insulin resistance in lactating dairy cows to benefit the homeorhetic shift that occurs during the transition from gestation to lactation to support milk production. However, future

research in dairy cows during early lactation is needed to characterize the potential role of rbIL-8 in altering the insulin action.

CONCLUSION

In summary, systemic rbIL-8 administration induces peripheral insulin resistance in bull Holstein calves, and this resistance persist after treatment, induces systemic inflammation, and alters blood metabolites, and blood cell counts. Further research in lactating cows is needed to evaluate the effect of rbIL-8 on the homeorhetic adaptations that occur after parturition to support lactation, particularly on the development of insulin resistance.

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CHAPTER 4: Effects of recombinant bovine interleukin-8 (rbIL-8) treatment on health, metabolism, and milk production in Holstein cattle IV: insulin resistance, dry matter intake, and blood parameters

ABSTRACT

We have shown in 2 independent studies that cows who received recombinant bovine interleukin-8 (**rbIL-8**) administered intrauterine shortly after parturition have a significant and long-lasting increase in milk yield. In the present study, we hypothesized that the increased milk production associated with rbIL-8 treatment is a consequence of increased postpartum dry matter intake (DMI) and orchestrated homeorhetic changes that prioritize milk production. Cows were enrolled into 1 of 3 treatment groups: those assigned to the control group (**CON**, n= 70) received an intrauterine (**IU**) infusion of 500 mL of Dulbecco's phosphate buffered saline (**DPBS**) solution and 1 mL of DPBS solution intravenously (**IV**; jugular vein), those assigned to the rbIL-8 IV group (**rbIL8-IV**, n= 70) received an IV injection of 70 µg of rbIL-8 and 500 mL of DPBS solution IU, and cows assigned to the rbIL-8 IU group (**rbIL8-IU,** n= 70) received an IU infusion with 500 µg of rbIL-8 diluted in 499.5 mL of DPBS solution and 1 mL of PBS solution IV. Animals were housed in tie-stall from calving to 30 DIM to measure DMI. Blood samples were collected daily from calving to 7 DIM and weekly until 28 DIM. Insulin resistance was evaluated using intravenous glucose tolerance test (**IVGTT**) and intravenous insulin challenge test (**IVICT**) in a subgroup of cows (n = 20/treatment) at 10 and 11 DIM, respectively. Additionally, liver biopsy samples were taken at 14 DIM from the same subgroup of cows to measure triglycerides (**TG**) levels and cell proliferation and apoptosis. Cows treated with rbIL8-IU produced more milk (CTR = 36.9 ± 1.5; rbIL8-IU = 38.5 ± 1.5; rbIL8-IV = 36.6 ± 1.5 kg/d), ECM (CTR = 42.9 ± 0.9; rbIL8-IU = 46.1 ± 0.8; rbIL8-IV =43.7 ± 0.9 kg/d), and FCM (CTR = 44.3 ± 0.9; rbIL8-IU = 47.8 ± 0.9; rbIL8-IV = 45.2 ± 0.9 kg/d) yields when compared with CTR cows, and no differences were observed between rbIL8-IV and CTR cows. The administration of rbIL8-IU significantly increased DMI compared with CTR (CTR = 18.8 ± 0.3; rbIL8-IU = 19.9 ± 0.3; rbIL8-IV = 19.3 ± 0.3 kg/d). Recombinant bIL-8 treatment did not affect glucose, insulin or fatty acids (i.e. IVICT only) concentrations and their area under the curve in response to IVGTT and IVICT when compared with CTR. Moreover, rbIL-8 treatment administered IU or IV increased liver TG levels. Additionally, cows treated with rbIL8-IU had lower odds of

developing hyperketonemia (OR = 0.46, 95% CI: 0.21 to 0.99), lower odds of clinical ketosis and displaced abomasum combined (OR = 0.17, 95% CI: 0.03 to 0.89), and lower odds of diseases combined (OR = 0.43, 95% CI: 0.21 to 0.86) when compared with CTR. We conclude that the administration of rbIL8-IU increases DMI, milk production, FCM, and ECM while improving the overall health during the postpartum period. This study supports the use of rbIL-8 administered IU shortly after calving to improve health and production responses in lactating cows.

Keywords: Interleukin-8, dry mater intake, metabolism, milk production.

INTRODUCTION

Dairy cows face numerous metabolic adaptations in the transition from late gestation to early lactation. During this transition, homeorhetic mechanisms allow dairy cows to produce copious amount of milk without compromising their own health and welfare (Bauman and Currie, 1980; Baumgard et al., 2017). However, the failure to adapt can lead to low milk production, health disorders, and poor reproductive performance (Dubuc et al., 2011; Chapinal et al., 2012; Bicalho et al., 2017). Thus, a successful lactation is dependent on the cow's ability to orchestrate adaptions that coordinate body tissues metabolism and nutrient trafficking to maximize milk synthesis and health.

Several hormones, such us insulin and growth hormone (**GH**), are considered as key regulators of nutrient partitioning (Bell, 1995; Lucy et al., 2009). At the onset of lactation, blood insulin levels rapidly decrease to then slowly increase during the progression of lactation (Zinicola and Bicalho, 2018). Conversely, blood GH concentrations significantly increase to then decrease as lactation progresses (Lucy et al., 2009). As a result, a series of adaptations such us increased lipolysis, increased gluconeogenesis, and decreased uptake of glucose by peripheral tissues ensure adequate nutrient supply to support lactation (Bell, 1995). Furthermore, transient insulin resistance allows for glucose to be spared by peripheral tissues and directed toward

synthesis of lactose in the mammary gland (Bell and Bauman, 1997; De Koster and Opsomer, 2013).

We have demonstrated that a single intrauterine administration of bovine recombinant interleukin 8 (**rbIL-8**) within 12 h of parturition significantly increased milk production, ECM, and FCM yields during the first 6 months of lactation (Zinicola et al. 2018a). Interestingly, we also observed that intrauterine rbIL-8 treatment was associated with a lower incidence of hyperketonemia (**HYK**). These results suggested that the production responses following rbIL-8 administration might be associated with an improved metabolic health (Zinicola et al. 2018a).

Interleukin 8 (**IL-8**) has been associated with insulin resistance (Fujishiro et al., 2003; Kobashi et al., 2009; Hardy et al., 2011). In fact, we have demonstrated that systemic administration of rbIL-8 in Holstein calves induces insulin resistance (Zinicola et al., 2018b). Moreover, IL-8 belongs to a subset of chemokines associated with angiogenic properties (Onuffer and Horuk, 2002), and mitogenic and antiapoptotic effects (Colletti et al., 1998; Hogaboam et al., 1999; Osawa et al., 2002).

Furthermore, we suggested that rbIL-8 treatment might exacerbate the un-coupling of the GH/insulin-like growth factor-I (**IGF-1)** axis that postpartum cows experience during early lactation. Cytokines are known to alter the GH signaling via suppressors of cytokine signaling (**SOCS**) proteins production, which will result in lower production of IGF-1 and thus exacerbating the un-coupling of the GH/IGF-1 axis (Bazan, 1989; Stevenson et al., 2004; Rico-Bautista et al., 2006). As a result, dairy cows might benefit from the well-known effects of GH on homeorhesis, which will lead to more milk production (Bauman and Vernon, 1993; Bell and Bauman, 1997; Baumgard et al., 2017). In contrast with our hypothesis, we have demonstrated that systemic administration of rbIL-8 in male Holstein calves does not alter GH and IGF-1 plasma concentrations (Zinicola et al., 2018b). However, the possible effect of rbIL-8 treatment on blood GH and IGF-1 levels in lactating animals is unknown.

We have proposed that the potential underlying mechanisms responsible for the increased milk production observed in Holstein cows following rbIL-8 treatment might involve one or

more of the following biological processes: (1) insulin resistance; (2) dysregulation of the GH/IGF-1 axis; (3) increased hepatocyte proliferation and decreased apoptosis; (4) and increased DMI. The primary objectives of the current study were to evaluate whether rbIL-8 treatment within 12 h of parturition would increase milk production through effects on insulin resistance, DMI, liver cell proliferation and apoptosis, and/or by promoting the un-coupling of the GH/IGF-1 axis.

MATERIALS AND METHODS

Ethics Statement

The research protocol was reviewed and approved by the Cornell University Institutional Animal Care and Use Committee (protocol number 2016-0065). The methods were carried out in accordance with the approved guidelines.

Animals, Housing, and Experimental Design

The study was conducted at the Cornell Dairy Teaching and Research Center, located in Harford, NY. The experimental farm has the capacity to house 80 cows in a tie-stall barn equipped with sawdust bedding, exhausters, *ad libitum* access to water, and completely separated feed bunks that allows accurate evaluation of feed intake. Additionally, ~500 lactating cows are housed in a free-stall barn, sand bedding, fans, and *ad libitum* access to feed and water. Animals were housed at the tie-stall facilities from calving to 30 DIM. At 31 DIM, cows were moved to the free-stall facilities until the end of the study period (180 DIM). From 0 to 30 DIM, cows were fed once daily (between 0630 and 0730 h) at 110% of expected consumption and individual feed intake was measured by weighing the amount of feed offered and refused daily. Total mixed ration was sampled weekly and evaluated for DM content (55 °C for 48 hours). Weekly dried TMR samples were ground to pass the 2-mm screen of a Wiley mill (Arthur H. Thomas, Philadelphia, PA), and monthly composites of dried TMR samples were sent to a commercial laboratory (Dairy One Cooperative Inc., Ithaca, NY) for chemical composition analysis;

ingredients and chemical composition of the ration are presented in tables 1 and 2, respectively. Individual feed intakes were recorded daily and corrected for weekly DM contents; weekly averages of DMI were used for data analysis. From 31 to 180 DIM, cows were fed twice daily in the free-stall barn equipped with headlock stations. Cows were milked thrice daily; milk weights were recorded at each milking. Nulliparous animals were moved to the tie-stall barn 21 ± 3 d from the anticipated calving date and were exercised 3 times per wk until calving to acclimate them to tie-stalls. Multiparous cows were moved to the tie-stall barn within 12 h of parturition.

Cows received first service timed AI (TAI) at 64 to 70 DIM after synchronization of ovulation with the Double-Ovsynch protocol (GnRH, 7 d later PGF, 3 d later GnRH, 7 d later GnRH, 7 d later PGF, 1 d later PGF, 32 h later GnRH). After first service, cows were inseminated after detection of estrus through a combination of visual observation and automated monitoring of physical activity (HR-Tags, SCR Dairy, Madison, WI). Pregnancy status was determined 32 ± 3 d after AI by transrectal ultrasonography using a 7.5 MHz transducer (Ibex, E.I. Medical, Loveland, CO). After determination of pregnancy status, nonpregnant cows were classified based on the ovarian structures present to receive the Short-Resynch or CIDR-Synch protocol as described in (Wijma et al., 2018). Briefly, cows with a corpus luteum (CL) ≥ 15 mm and a follicle ≥ 10 mm received the Short-Resynch protocol (PGF, 1 d later PGF, 32 h later GnRH-16 to 18 h TAI) whereas cows without a CL ≥ 15 mm, a follicle ≥ 10 mm, or both received CIDR-Synch (GnRH plus CIDR, 7 d later PGF and CIDR removal, 1 d later PGF, 32 h later GnRH-16 to 18 h TAI). Cows diagnosed pregnant 32 ± 3 d after were reexamined 60 ± 3 d after AI to re-confirm pregnancy. Cows were considered pregnant for the analysis of time to pregnancy if confirmed pregnant at the second examination.

A total of 210 cows (primiparous, n = 90; multiparous, n = 120) were enrolled between September 2017 and April 2018. Treatment allocation was done by parity (primiparous/multiparous) in two separate randomization sheets within 12 h of parturition. The experimental design was a randomized complete block design with one-way treatment structure. The blocking factor was based on order of calving (order of enrollment). The

allotment also indicated the subset of cows (primiparous, n = 10/treatment; multiparous, n = 10/treatment) where peripheral response to insulin and glucose disappearance and liver tissue samples were evaluated/collected. The subset of cows was randomly selected to ensure that they were distributed equally across the duration of the study. The random treatment and blocks were generated using the random number generator function (SAS RANUNI function) of SAS (version 9.4; SAS/STAT, SAS Institute Inc., Cary, NC). If the following conditions were encountered at enrollment cows were excluded from the study: twins, lameness, milk fever, displaced abomasum (**DA**), mastitis, and severe injury/trauma.

At the first signs of delivery, cows were moved to individual maternity pens where trained farm personnel assisted with parturition as needed. Cows were enrolled in 1 of 3 treatment groups: those assigned to the control group (**CON**, n= 70) received an intrauterine (**IU**) infusion of 500 mL of Dulbecco's phosphate buffered saline (**DPBS**, #14190-144; Gibco, Paisley, UK) solution and 1 mL of DPBS solution intravenously (**IV**; jugular vein), those assigned to the rbIL-8 IV group (**rbIL8-IV**, n= 70) received an IV injection of 70 µg of rbIL-8 and 500 mL of DPBS solution IU, and cows assigned to the rbIL-8 IU group (**rbIL8-IU,** n= 70) received an IU infusion with 500 µg of rbIL-8 diluted in 499.5 mL of DPBS solution and 1 mL of PBS solution IV. Recombinant bIL-8 was provided by Zoetis (Zoetis Animal Health, Kalamazoo, MI).

Animal Sampling

Blood samples were collected after feeding (0800 to 1000 h) from coccygeal vessels using 10-mL vacutainer lithium heparin blood collection tubes (Greiner Bio-One, Monroe, NC) for plasma separation, and 3-mL vacutainer K_2 EDTA tubes (BD Vacutainer, Franklin Lakes, NJ) for complete blood cell counts and genomic testing (CLARIFIDE, Zoetis Animal Health, Kalamazoo, MI). Sampling was done daily from 0 (before treatment) to 7 DIM and weekly from 14 to 28 DIM. After collection, samples were immediately placed on ice. Samples were used for hemogram determination using a Vet hemogram instrument (Heska – Hemature^{tm}, Loveland,

CO), and plasma was obtained within 1.5 h of blood collection by centrifugation at 2,000 × *g* for 15 min at 4 °C, and frozen at -80 °C. Plasma concentrations of aspartate aminotransferase (**AST**), alanine aminotransferase (**ALT**), alkaline phosphatase (**ALP**), gamma glutamyl transferase (**GGT**), BSA, total bilirubin, total protein, creatinine, lactate, cholesterol, urea, glucose, BHB, and fatty acids were determined using an automated clinical chemistry analyzer (Daytona, Randox Laboratories Ltd., Kerneysville, WV), using reagents provided by Randox. Plasma Insulin concentration was determined using a commercial ELISA kit for bovine insulin (Bovine Insulin ELISA, ALPCO, Salem, NH). Following the procedures previously described, a double RIA was used for measuring the plasma concentrations of IGF-1 (Butler et al., 2003; Butler et al., 2004) and GH (Plaut et al., 1987; Rosemberg et al., 1989). h-IGF-1 receptor grade (lot# EBB-CO1) from Gropep (Adelaine, Australia) was used for iodination and standards. Primary antibody anti-h-IGF1-rabbit (#AFP4892898) was obtained from Dr. A.F. Parlow at the National Hormone and Pituitary Program and diluted 1:55k for the assay. For the bGH assay, standards and iodinated tracer were made from Upjohn Growth Hormone, Lot#12 code #77-001. NIDDK-anti-oGH-2 (rabbit) AFPC0123080 was added at 1:35k as the primary antibody. For both assays the second antibody used for precipitation was sheep anti-rabbit gamma globulin supplied by Dr. W.R.Butler, Cornell University.

For each cow, rectal temperature (**RT**) was measured at the blood collection time points. Body condition score (**BCS;** (Edmonson et al., 1989)) and body weight (**BW**) were recorded weekly after the morning milking from calving to 30 DIM, and every other week from 45 to 90 DIM. Vaginal discharge was evaluated in all study cows at 3, 7, 14, and 28 DIM using the Metricheck device (Metricheck, SimcroTech, Hamilton, New Zealand). Additionally, milk samples were collected for each cow into tubes containing 2-bromo-2-nitro-1,3-propanediol preservative during the morning milking daily from 1 to 6 DIM, and weekly from 7 to 28 DIM. Milk samples were submitted to a commercial laboratory (Dairy One Cooperative Inc., Ithaca, NY) for evaluation of percentage fat, protein, lactose, and SCC.

Evaluation of the Peripheral Response to Insulin and Glucose Disappearance

The peripheral response to glucose and insulin was evaluated using intravenous glucose tolerance test (**IVGTT**) and intravenous insulin challenge test (**IVICT**) in a subgroup of cows (n = 20/treatment). Long-term 16Ga catheters (Mila International Inc., Florence, KY) were placed into the jugular vein at 9 DIM. Intravenous GTT and IVICT were performed on DIM 10 and 11, respectively. Cows were fasted for 3 hour prior to each test, and tests were performed right after the morning milking. For the IVGTT, cows were infused with 0.25 g/kg of BW of glucose (dextrose 50%, wt/vol; Phoenix Scientific Inc., St. Joseph, MO) followed by 20 mL of sterile saline solution. For the IVICT, cows were infused with 0.2 IU of insulin/kg of BW (Sigma-Aldrich, St. Louis, MO) followed by 20 mL of sterile saline solution. Blood was sampled at -15, 0, 10, 20, 30, 45, 60, 90, and 120 minutes relative to glucose or insulin infusion. In both tests, the catheters were flushed between sampling points with sterile saline solution containing sodium heparin (Sagent, Schaumburg, IL) to avoid clotting. The initial 3 mL of blood drawn from the catheter at every sampling point was discharged before the sample was collected to avoid dilution. Blood was collected into evacuated tubes containing lithium heparin (Greiner Bio-One, Monroe, NC). Samples were placed on ice immediately and centrifuged within 1 hour of collection as described above. Concentrations of glucose, insulin, and fatty acids (i.e. IVICT only) were evaluated as described above.

Liver Biopsy

Liver tissue was taken from a subgroup of cows (n = 20/treatment) at 14 DIM. Briefly, cow's hair was clipped on the right side at the 11th intercostal space. Ultrasonography (Ibex Pro, E.I. Medical Imaging, Loveland, CO) was performed to confirm the location of the liver and to avoid large hepatic blood vessels. The skin was disinfected thrice with iodine (7.5% v/v; VetOne, Boise, ID) and once with ethanol (70% v/v; VetOne, Boise, ID). Local anesthesia was performed using lidocaine (2% lidocaine hydrochloride, MWI, Boise, ID). Approximately 10 minutes after the injection of lidocaine, the skin was punctured with a scalpel and a stainless-

steel trocar (31 cm long and 7.5 mm in diameter) was introduced to the abdominal cavity directing the trocar toward the left elbow and ~2 g of liver tissue was harvested. The incision was closed with surgical staples (3M Precise, St. Paul, MN) and coated with aluminum spray bandage. Liver tissue samples were blotted on sterile gauze to remove blood and fixed in 10% neutral buffered formalin for subsequent paraffin embedding and histological evaluation.

Analysis of Liver Cell Proliferation and Apoptosis

Liver cell apoptosis

Four-µm–thick sections of formalin-fixed/paraffin-embedded liver biopsies were used for TUNEL analysis. After deparaffinization in xylene and rehydration in graded ethanol, antigen retrieval was performed by incubating slides in heated citrate buffer (0.01 mol/L, pH 6.0) for 8 minutes following by cooling down in tap water for 10 minutes. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in distilled water for 10 minutes. Then, the endogenous avidin/biotin were blocked with avidin/biotin blocking kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. For easy handling and minimizing variation, slides of multiple cases were processed in a batch with MicroProbe System (Fisher Scientific, Hampton, NH) and PBST (0.05% Tween 20) was used for washing throughout the procedure. After washing in PBST for two times (5 min each), apoptotic cells were detected using DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI). The sections were incubated with biotinylated nucleotide mix for 1.5 h at 37 °C in a humid chamber. Nova Red (Vector Laboratories, Burlingame, CA) was used as chromogen to visualize antigen localization, and the sections were lightly counterstained with hematoxylin. Bovine tissue microarray with known apoptosis were used for protocol validation. Immunohistochemistry results were examined by Olympus AX 70 compound microscope equipped with MicroFire camera and PictureFrame for image processing and capture (Optronics, Goleta, CA).

Liver cell proliferation

Formalin fixed paraffin embedded tissue blocks were processed for Ki-67 antigen detection by Immunohistochemistry staining at the Animal Health Diagnostic Center at Cornell University using an automated immunohistochemistry stainer (Leica Bond Max, Leica Biosystems, Buffalo Grove, IL). All steps for the immunohistochemistry staining were performed directly on the stainer. Slides were dewaxed (#AR9222, Leica Bond Dewax solution) and then processed for heat epitope retrieval for 30 minutes (#AR9961, Leica Bond Epitope Retrieval solution 1) followed by incubation with the primary antibody, mouse monoclonal anti-human KI67, clone MIB-1 (#M7240, DakoCytomation, Carpinteria, CA, USA) for 60 minutes. Next Powervision-AP-Anti-Mouse IgG (#PV6110, Leica Powervision Product) was applied to the slides for 30 minutes followed by the Red Detection (#DS9390, Leica Bond Refine Red Detection Kit) for 15 minutes. Lastly, hematoxylin (#DS9390, Leica Bond Refine Red Detection Kit) was applied to the slides for 5 minutes and cover slipped.

Quantification of immunohistochemistry

Quantification of apoptotic cells and Ki-67 antigen expressing cells was done using a proprietary algorithm (IHC Nuclear, v1, Leica Aperio, Gmbh) with settings previously optimized using a tissue microarray of 20 normal bovine tissues, including liver.

Liver Tissue Triglyceride Measurement

Liver triglycerides (**TG**) was measured using the Folch extraction method (Folch et al., 1957) followed by a modified colorimetric assay based on Hantzsch condensation for estimating serum triglyceride (Fletcher, 1968; Foster and Dunn, 1973). Briefly, samples were weighted (0.15 g) and extracted using chloroform:methanol (2:1 v/v). Following homogenization and centrifugation, extracted samples were mixed with aluminum oxide and saponified using 5% KOH diluted in isopropanol-ddH_2O (40:60 v/v). Sodium metaperiodate and acetylacetone reagents were added and samples were read at 405 nm. Fatty liver was categorized based on the

percentage of liver TG into normal liver (< 1% of liver TG), and mild (1-5 % of liver TG), moderate (5-10% of liver TG), and severe fatty liver (> 10% of liver TG), as previously described (Bobe et al., 2004).

Disease Definitions

Dystocia was defined as a calving that required assistance from farm personnel. Retained placenta (**RP**) was defined as cows that failed to release the fetal membranes within 24 h after calving (Kelton et al., 1998). Diagnosis of metritis, puerperal metritis, and endometritis were performed by research personnel based on evaluation of vaginal mucus retrieved using a Metricheck device. Vaginal discharge was scored using a modified 0 to 5 scale (0 = no secretion material retrieved; 1 = clear mucus; 2 = clear mucus with flecks of pus; 3 = mucopurulent discharge containing < 50% of pus; 4 = mucopurulent discharge containing ≥ 50% of pus; 5 = watery, red-brown, fetid vaginal discharge). Cows with a score = 5 at 3, 7, or 14 DIM were considered to have clinical metritis. Puerperal metritis was defined as cows having clinical metritis with RT > 39.5°C (Sheldon et al., 2006). Cows with a score ≥ 3 at 28 DIM were considered to have clinical endometritis. Displaced abomasum diagnosis was made by researchers and confirmed by veterinarians. Lameness was defined as cows with clinical manifestation of abnormal locomotion detected by researchers and farm personnel. Clinical ketosis (**CK**) was detected by researchers and farm personnel and defined as cows with decreased milk production, reduced feed intake, low rumen fill, weakness, dullness, depression, with/without ketone odor in breath, and high blood concentration of BHB. Hyperketonemia (**HYK**) was defined as plasma BHB concentration ≥1.4 mmol/L from blood samples collected from 1 to 28 DIM. Clinical mastitis was evaluated during the first 30 DIM and defined as the presence of abnormal milk, such as watery appearance, flakes and clots in milk during forestripping detected at each milking by trained farm employees. Subclinical mastitis (**SCM**) was defined as cows detected with a SCS ≥ 4 at 7, 14, 21, and/or 28 DIM.

Calculations and Data Analysis

Energy balance (**EBAL**) was calculated based on the weekly energy intake: NE_L intake = (weekly DMI × diet energy density); maintenance requirements: NE_L for maintenance = (0.08 × $BW^{0.75}$); and milk energy output: NE_L for milk production = {weekly milk yield × [(0.0929 × fat %) + (0.0563 × protein %) + (0.0395 × lactose %)]}. Energy balance was calculated as follows: NE_L balance (Mcal) = NE_L intake – (NE_L for milk production + NE_L for maintenance). Weekly ECM yield was calculated from 0 to 30 DIM as follows: ECM (kg) = [(0.3246 × milk yield) + (12.86 × fat yield) + (7.04 × protein yield)]. Weekly 3.5% FCM yield was calculated from 0 to 30 DIM as follows: FCM (kg) = [(0.4324 × milk yield) + (16.216 × fat yield)]. Production efficiency was calculated based on milk yield, ECM, and DMI as follows: milk yield efficiency (kg) = (milk yield/DMI); ECM yield efficiency (kg) = (ECM/DMI). Linear somatic cell score was calculated as follows: SCS = {[ln (SCC / 100 × 10^3) /0.693147] + 3}.

Insulin sensitivity was evaluated using the following insulin sensitivity indexes: quantitative insulin sensitivity check index (**QUICKI**) = {1/[log insulin (μU/mL) + log glucose (mg/dL)]}, according to (Katz et al., 2000); revised QUICKI (**RQUICKI**) = {1/[log glucose (mg/dL) + log insulin (μU/mL) + log fatty acids (mmol/L)]}, according to (Perseghin et al., 2001; Holtenius and Holtenius, 2007); revised QUICKI including BHB (**$RQUICKI_{BHB}$**) = {1/[log glucose (mg/dL) + log insulin (μU/mL) + log fatty acids (mmol/L) + log BHB (mmol/L)]}, according to (Balogh et al., 2008); and the homeostatic model assessment of insulin resistance (**HOMA-IR**) = {[glucose (mmol/L) × insulin (μU/mL)]/22.5}, according to (Muniyappa et al., 2008). Moreover, plasma concentrations of glucose, insulin, and fatty acids (i.e. IVICT only) from IVGTT and IVICT were used to create response curves. The positive incremental area under the curve (**AUC**) at 120 min after glucose or insulin infusion of glucose, insulin, and fatty acids was calculated in SAS, correcting for baseline based on the trapezoidal method as previously described (Cardoso et al., 2011).

Descriptive statistics were performed in JMP Pro 11 (SAS Institute Inc., NC), using the ANOVA and chi-square functions for continuous and categorical data, respectively. Collinearity

among predictor variables was assessed using the multivariate procedure in JMP. We detected a collinearity issue between the variables parity and BW at enrollment ($r = 0.82$; $P < 0.001$). We offered to the models the variables parity and BW at enrollment separately, and the one that yielded the minimum Akaike information criterion (AIC) was chosen. Therefore, for milk production, ECM, FCM, DMI, production efficiency, and EBAL analyses, BW at enrollment was included in all models. Enrollment block was included as random effect. For analyses of repeated measurements, we used general mixed linear models with the MIXED procedure of SAS (version 9.4; SAS/STAT, SAS Institute Inc., Cary, NC). The independent variables offered to the models were: treatment, time, BW at enrollment, BCS at enrollment, RT at enrollment, dystocia, stillbirth, subset (cows that underwent to IVGTT/IVICT and liver biopsy) time from calving to treatment (enrollment), placenta present at enrollment, and previous days carry calf. Two-way and three-way interaction terms between independent variables were offered to the models. For hemogram parameters, metabolites, and hormones concentrations, baseline values were treated as covariate variables. Normality and homoscedasticity of residuals were assessed using residual plots. Continuous data with residuals not normally distributed were $\log_{10}$ transformed. Several covariance structures were tested (unstructured, autoregressive 1, compound symmetry), and the one with the minimum AIC was chosen. For all models, Dunnett's significance test for multiple comparisons was used. Data are reported as LSM ± SEM unless otherwise stated.

The effect of treatment on binary response variables was analyzed by logistic regression using the GLIMMIX procedure of SAS and the fixed effects of treatment, parity, BCS at enrollment, RT at enrollment, dystocia, stillbirth, time from calving to treatment, placenta present at enrollment, and the interaction term treatment by parity were included as independent variables in the statistical models. Dunnett's significance test for multiple comparisons was used. A categorical variable named "diseases combined" was created to evaluate the effect of treatment on the proportion of cows affected by common diseases during the first 30 DIM; the

following diseases were combined for analysis: RP, metritis, puerperal metritis, endometritis, DA, CK, lameness, CM, and SCM. In addition, CK and DA were also combined for analysis.

The effect of treatment on the hazard of pregnancy and herd removal during the first 180 DIM were analyzed by Cox's Proportional Hazard using the PHREG procedure in SAS. Variables offered to the models included treatment, parity, BCS at enrollment, dystocia, stillbirth, time from calving to treatment, placenta present at enrollment, and interactions. To illustrate the effect of treatment on reproductive performance and herd removal, Kaplan–Meier survival analysis was carried out using MedCalc version 11.5.1.0 software (MedCalc Software).

For all models, variables and their respective interaction terms were removed from the models in a backward stepwise elimination procedure when $P \leq 0.15$. Significances were considered when $P \leq 0.05$ or a trend if $0.05 < P \leq 0.10$.

RESULTS

Lactation Performance, Dry Matter Intake, and Energy Balance

To test for the genetic merit of milk production between treatment groups, genetic differences in total pounds of milk produced during a 305-day lactation (CLARIFIDE test) was evaluated. No significant differences ($P = 0.65$) were observed among groups.

The effects of rbIL-8 treatment on weekly milk averages (first 25 wk of lactation), ECM, and FCM yields (first 4 wk of lactation) are presented in Table 4.3 and Figure 4.1-2. Cows treated with rbIL8-IU produced 1.6, 3.1, and 3.5 kg/d more milk ($P = 0.03$), ECM ($P = 0.006$), and FCM ($P = 0.004$) yields when compared with CTR cows; no differences were observed between the rbIL8-IV and CTR groups.

Milk fat, protein, and lactose percentage were not affected by treatment (Table 4.3). However, cows treated with rbIL8-IU produced 0.15 and 0.08 kg/d more fat ($P = 0.007$) and lactose ($P = 0.07$) respectively when compared with control cows, but no differences were observed between the rbIL8-IV and CTR group. Milk protein yield and SCS were not affected by rbIL-8 treatment (Table 4.3).

Dry matter intake during the first 4 wk of lactation is presented in Table 4.3 and Figure 4.2. We observed that cows treated with rbIL8-IU consumed 1.1 kg/d more DM ($P = 0.03$) when compared with CTR cows, but no differences were observed between the rbIL8-IV and CTR groups. Ratios of milk production and ECM to DMI, and EBAL were not affected by treatment (Table 4.3).

Plasma Metabolites, Hepatic Enzymes, and Hormones Concentrations

Plasma concentrations of fatty acids, BHB, glucose, cholesterol, total bilirubin, urea, lactate albumin, total protein, ALT, AST, GTT, ALP, and haptoglobin were not affected by treatment (Table 4.4, Figure 4.3, and Supplementary Figure 4.1-2). Moreover, treatment did not affect plasma levels of insulin, GH, and IGF-1 (Table 4.4 and Figures 4.3-4).

Body Condition Score and Body Weight loss

The effects of treatment on BCS and BW loss during the first 90 d of lactation are illustrated in Figure 4.5. No differences were observed between treatment groups.

Liver Triglycerides Levels, Cell Proliferation, and Cell Apoptosis

Liver TG levels were affected ($P = 0.01$) by rbIL-8 treatment. Cows treated with rbIL8-IU or rbIL8-IV had higher liver TG concentrations when compared with CTR cows (Figure 4.6). Liver cell proliferation was not affected by treatment. Cows treated with rbIL8-IV cows had ($P = 0.003$) a higher number of apoptotic cells than CTR cows (Figure 4.6). However, the ratio liver cell apoptosis to cell proliferation was not affected ($P = 0.31$) by treatment (Figure 4.6). Furthermore, no differences were observed between treatment groups when cows were classified based on TG levels in mild and moderate fatty liver (no cows were detected with normal or severe fatty liver; Figure 4.6).

IVGTT, IVICT, and Insulin Sensitivity Indexes

For the IVGTT, glucose and insulin AUC were not affected by treatment (Figure 4.7A). For the IVICT, glucose and fatty acids AUC were not affected by treatment (Figure 4.7B). Moreover, QUICKI, RQUICKI, $RQUICKI_{BHB}$, and HOMA-IR did not differ between treatment groups (Figure 4.8).

Hemogram Parameters and Rectal Temperature

Results of white blood cells, PMNs, lymphocytes, monocytes, red blood cell numbers, and percentage hematocrit are illustrated in Supplementary Figure 4.3. No differences were observed on white blood cells, PMNs, lymphocytes, and monocytes numbers between treatment groups. However, rbIL-8 treatment did affect ($P = 0.002$) red blood cells numbers. Relative to CTR cows, rbIL8-IU cows had lower red blood cells numbers and no differences were observed between rbIL8-IV and CTR groups. Additionally, we observed that cows treated with rbIL8-IU tended to have lower percentage hematocrit when compared with CTR cows and no differences were observed between rbIL8-IV and CTR cows. Treatment did not affect ($P = 0.34$) RT during the first 4 weeks postpartum (CTR = 38.6 ± 0.03; rbIL8-IU = 38.7 ± 0.03; rbIL8-IV = 38.7 ± 0.03 °C).

Health Disorders

Treatment did not alter the incidence of RP, metritis, puerperal metritis, endometritis, DA, CK, CM, SCM, and lameness (Table 4.5). Cows treated with rbIL8-IU had lower odds of developing HYK when compared with CTR cows (Table 4.5). Moreover, rbIL8-IU cows had lower odds of developing CK and DA combined than CTR cows (Table 4.5). Additionally, cows treated with rbIL8-IU had lower odds of being diagnosed with 1 or more diseases when compared with CTR cows (Table 4.5).

Removal From the Herd and Reproductive Performance

Treatment did not alter ($P = 0.23$) the hazard of culling during the first 180 DIM (rbIL8-IU, HR: 0.79, 95% CI: 0.54 to 1.63; rbIL8-IV, HR: 0.94, 95% CI: 0.65 to 1.38; Supplementary Figure 4.4A). Moreover, treatment did not alter ($P = 0.47$) the hazard of pregnancy during the first 180 DIM (rbIL8-IU, HR: 0.79, 95% CI: 0.54 to 1.63; rbIL8-IV, HR: 0.94, 95% CI: 0.65 to 1.38; Supplementary Figure 4.4B). The median calving-to-conception interval for CTR, rbIL8-IU, and rbIL8-IV was 70.5, 86, and 80 d, respectively.

Table 4.1. Ingredients (DM basis) of postpartum diet.

Ingredient	% of DM
Alfalfa hay	3.60
Wheat straw	0.90
Canola meal	4.50
Ear corn, high-moisture	10.80
Mixed-legume grass silage	10.80
Corn Silage	43.55
Energy Booster 100[1]	1.35
Celmanex[2]	0.22
Amino Plus[3]	7.37
Dried molasses	3.74
Citrus pulp	3.51
Soybean hulls	2.25
Blood meal	1.68
Fermenten[4]	0.77
Urea	0.45
Megalac R[5]	0.36
Ethyl-cellulose RPM[6]	0.04
Na bicarbonate	1.49
MIN AD[7]	0.53
Dynamate[8]	0.53
Salt	0.53
Mineral mix[9]	0.22
Mono Ca phosphate	0.21
Ca Carbonate	0.21
Mg Oxide	0.27
Rumensin[10]	0.06
Vitamin E premix[11]	0.05

[1]Commercial fat source, Milk Specialties Global, Eden Prairie, MN.
[2]Yeast product. Church & Dwight Co., Inc. Ewing Township, NJ.
[3]Heat-treated soybean meal. Ag Processing Inc., Omaha, NE.
[4]Rumen fermentation enhancer. Church & Dwight Co., Inc. Ewing Township, NJ.
[5]Rumen bypass fat. Church & Dwight Co., Inc. Princeton, NJ.
[6]Ethyl-cellulose rumen-protected methionine, Evonik Nutrition and Care GmbH (Hanau-Wolfgang, Germany).
[7]Ca-Mg dolomite, Papillon Agricultural Company, Easton, MD.
[8]K Mg sulfate. The Mosaic Co., Plymouth, MN.
[9]Contains 25% of Ca, 6.5 % of Mg, 6.3% of S, 300 mg/kg of I, 250 mg/kg of Co, 4,000 mg/kg of Cu, 500 mg/kg of Fe, 13,300 mg/kg of Zn, 5,000 mg/kg of Mn, 125.7 mg/kg of Se, 581,818 IU/kg of vitamin A, 163.6 IU/kg of vitamin D, and 2,116 IU/kg of vitamin E.
[10]Premix contained 26,400 g/t of monensin. Elanco Animal Health, Greenfield, IN.
[11]Contains 44,000 kIU/kg of Vitamin E.

Table 4.2. Chemical composition (DM basis) of postpartum diet.

Energy and chemical compostion[1]	Mean	SD
NE_L, Mcal/Kg	1.64	0.02
NDF, %	30.47	1.02
NFC, %	39.81	1.37
ADF, %	19.73	1.50
Starch, %	24.92	1.53
Crud fat, %	3.42	0.31
CP, %	17.27	0.69
Ash, %	9.1	0.38
Lignin, %	2.97	0.40
Ca, %	0.88	0.04
P, %	0.39	0.01
K, %	1.69	0.16
Na, %	0.72	0.02
Mg, %	0.48	0.01
Fe, mg/kg	339	45.7
Zn, mg/kg	66	5.38
Cu, mg/kg	17	1.73
Mn, mg/kg	47	2.10
Mo, mg/kg	1.5	0.30

[1]Values represents averages of 8 monthly composited samples. Chemical composition analysis was performed by Dairy one Cooperative Inc., Ithaca, NY.

Table 4.3. Weekly milk yield for the first 25 weeks postpartum, and production and feed intake responses during the first 4 weeks of lactation of cows treated with recombinant bovine interleukin-8 intrauterine (rbIL8-IU, n = 70), intravenously (rbIL8-IV, n = 70), and controls (CTR, n = 70). Results are presented as LSM ± SEM.

Item	Treatment group			*P*-value[1]	
	CTR (LSM ± SEM)	rbIL8-IU (LSM ± SEM)	rbIL8-IV (LSM ± SEM)	CTR vs rbIL8-IU	CTR vs rbIL8-IV
Milk yield, kg/d[2]	36.9±1.5	38.5±1.5	36.9±1.5	0.05	0.99
ECM yield, kg/d	42.9±0.9	46.1±0.9	43.7±0.9	0.006	0.69
FCM yield, kg/d	44.3±0.9	47.8±0.9	45.2±0.9	0.004	0.64
Fat yield, kg/d	1.78±0.03	1.93±0.03	1.83±0.03	0.005	0.47
Protein yield, kg/d	1.23±0.02	1.27±0.02	1.23±0.02	0.15	0.84
Lactose yield, kg/d	1.56±0.08	1.64±0.08	1.57±0.08	0.07	0.89
Fat, %	5.25±0.06	5.26±0.06	5.28±0.06	0.99	0.92
Protein, %	3.76±0.04	3.75±0.04	3.75±0.04	0.90	0.93
Lactose, %	4.60±0.02	4.61±0.02	4.58±0.02	0.94	0.42
DMI, kg/d	18.8±0.3	19.9±0.3	19.3±0.3	0.03	0.39
EBAL, Mcal/wk	-15.2±0.6	-15.2±0.6	-14.6±0.6	0.99	0.66
Milk yield:DMI	2.0±0.03	2.0±0.03	1.9±0.03	0.79	0.29
ECM:DMI	2.4±0.03	2.4±0.03	2.3±0.03	0.99	0.41
SCS	3.7±0.14	3.4±0.14	3.6±0.15	0.48	0.91

[1]Dunnett's significance test for multiple comparisons was used.
[2]Milk yield for the first 25 weeks of lactation.
DMI = Dry matter intake.
EBAL = Energy balance.
SCS = Somatic cell linear score.

Table 4.4. Plasma metabolites, liver enzymes, and hormones concentrations during the first 4 weeks of lactation of cows treated with recombinant bovine interleukin-8 intrauterine (rbIL8-IU, n = 70), intravenously (rbIL8-IV, n = 70), and controls (CTR, n = 70).

Item	Treatment group			*P*-value[1]	
	CTR (LSM ± SEM)	rbIL8-IU (LSM ± SEM)	rbIL8-IV (LSM ± SEM)	CTR vs rbIL8-IU	CTR vs rbIL8-IV
Fatty acids, mmol/L	0.55±0.02	0.57±0.02	0.52±0.02	0.86	0.39
BHB, mmol/L	0.87±0.04[2]	0.86±0.04[2]	0.81±0.04[2]	0.95	0.31
Glucose, mg/dL	69.8±1.20	69.8±1.18	70.2±1.17	0.99	0.94
Cholesterol, mg/dL	114.3±1.79	117.2±1.77	116.3±1.79	0.41	0.64
Total bilirubin, mg/dL	0.24±0.007	0.24±0.007	0.23±0.007	0.94	0.32
Urea, mg/dL	26.1±0.41	25.9±0.41	26.1±0.40	0.92	0.99
Lactate, mg/dL	7.84±0.54	7.60±0.53	7.75±0.53	0.53	0.92
Albumin, g/dL	3.67±0.02	3.68±0.02	3.66±0.02	0.81	0.96
Total protein, mg/dL	7.57±0.05	7.61±0.05	7.51±0.05	0.78	0.60
ALT, U/L	25.2±0.83	25.0±0.82	24.9±0.81	0.80	0.60
AST, U/L	96.2±1.03[2]	95.6±1.03[2]	93.7±1.03[2]	0.95	0.43
GTT, U/L	21.0±0.61	21.4±0.59	20.5±0.61	0.86	0.77
ALP, U/L	56.6±1.26	57.1±1.23	59.5±1.26	0.95	0.20
Haptoglobin, OD_{450nm}	0.12±0.05[2]	0.12±0.04[2]	0.12±0.05[2]	0.97	0.99
Insulin, ng/mL	0.28±0.03[2]	0.28±0.03[2]	0.28±0.03[2]	0.84	0.71
GH, ng/mL	4.83±0.28	4.72±0.28	4.83±0.28	0.94	0.99
IGF-1, ng/mL	72.6±1.69	73.15±1.67	73.3±1.70	0.97	0.95

[1]Dunnett's significance test for multiple comparisons was used.
[2]log_{10} back-transformed concentrations.
BHB = β-hydroxybutyrate.
ALT = alkaline phosphatase.
AST = alanine aminotransferase.
GTT = gamma glutamyl transferase.
ALP = alanine aminotransferase.
OD = Optical density.
GH = Growth hormone.
IGF-1 = Insulin-like growth factor-I

Table 4.5. Incidence of periparturient diseases during the first 30 d postpartum of cows treated with recombinant bovine interleukin-8 intrauterine (rbIL8-IU, n = 70), intravenously (rbIL8-IV, n = 70), and controls (CTR, n = 70). Outcomes from logistic regression models are presented; control was considered as the reference group.

	% Incidence			OR[1] (95% CI)[2]		*P*-value		
Item	CTR	rbIL8-IU	rbIL8-IV	rbIL8-IU	rbIL8-IV	Trt[3]	Parity	Trt × parity
Retained placenta	4.29	2.66	7.25	0.65 (0.10, 4.10)	1.74 (0.40, 7.66)	0.48	0.89	0.93
Metritis	2.86	1.43	7.25	0.37 (0.03, 5.12)	2.94 (0.46, 18.6)	0.18	0.29	0.60
Puerperal metritis	0.00	0.00	2.90	-	-	-	-	-
Endometritis	4.48	7.25	8.82	1.95 (0.43, 8.85)	2.35 (0.53, 10.1)	0.52	0.65	0.47
Displaced abomasum	5.71	2.86	2.90	0.51 (0.08, 3.23)	0.54 (0.08, 3.34)	0.70	0.98	0.99
Clinical ketosis	7.14	1.43	4.35	0.21 (0.02, 2.09)	0.58 (0.11, 2.95)	0.40	0.97	0.98
Clinical ketosis + DA	11.4[a]	2.86[b]	4.35[a]	0.17 (0.03, 0.89)	0.25 (0.06, 1.13)	0.05	0.02	0.98
Hyperketonemia	56.0[a]	41.0[b]	52.0[a]	0.46 (0.21, 0.99)	0.98 (0.45, 2.16)	0.08	<0.01	0.53
Mastitis	5.71	7.14	4.35	1.27 (0.32, 4.97)	0.75 (0.16, 3.51)	0.78	0.96	0.89
Sub-clinical mastitis	38.6	28.6	33.3	0.55 (0.26, 1.15)	0.76 (0.37, 1.57)	0.28	0.90	0.85
Lameness	15.7	8.57	7.25	0.44 (0.14, 1.37)	0.36 (0.11, 1.16)	0.16	<0.01	0.98
Diseases combined[4]	58.6[a]	40.0[b]	50.7[a]	0.43 (0.21, 0.86)	0.66 (0.33, 1.32)	0.05	0.47	0.70

[1]OD, odds ratio.
[2]95 % confidence interval.
[3]Trt, treatment.
[4]The following diseases were used to evaluate the effect of treatment on the proportion of cows affected by diseases combined: retained placenta, metritis, puerperal metritis, endometritis, displaced abomasum, clinical ketosis, mastitis, sub-clinical mastitis, and lameness.
DA = displaced abomasum.
[a-b]Different superscripts within a row indicate a significant difference ($P \leq 0.05$); Dunnett's significance test for multiple comparisons was used.

Figure 4.1. Weekly milk yield for the first 25 weeks of lactation of cows treated with recombinant bovine interleukin-8 intrauterine (rbIL8-IU, n = 70), intravenously (rbIL8-IV, n = 70), and controls (CTR, n = 70). Dunnett's significance test for multiple comparisons was used: CTR vs rbIL8-IU $P = 0.05$; CTR vs rbIL8-IV $P = 0.99$. Results are presented as LSM ± SEM.

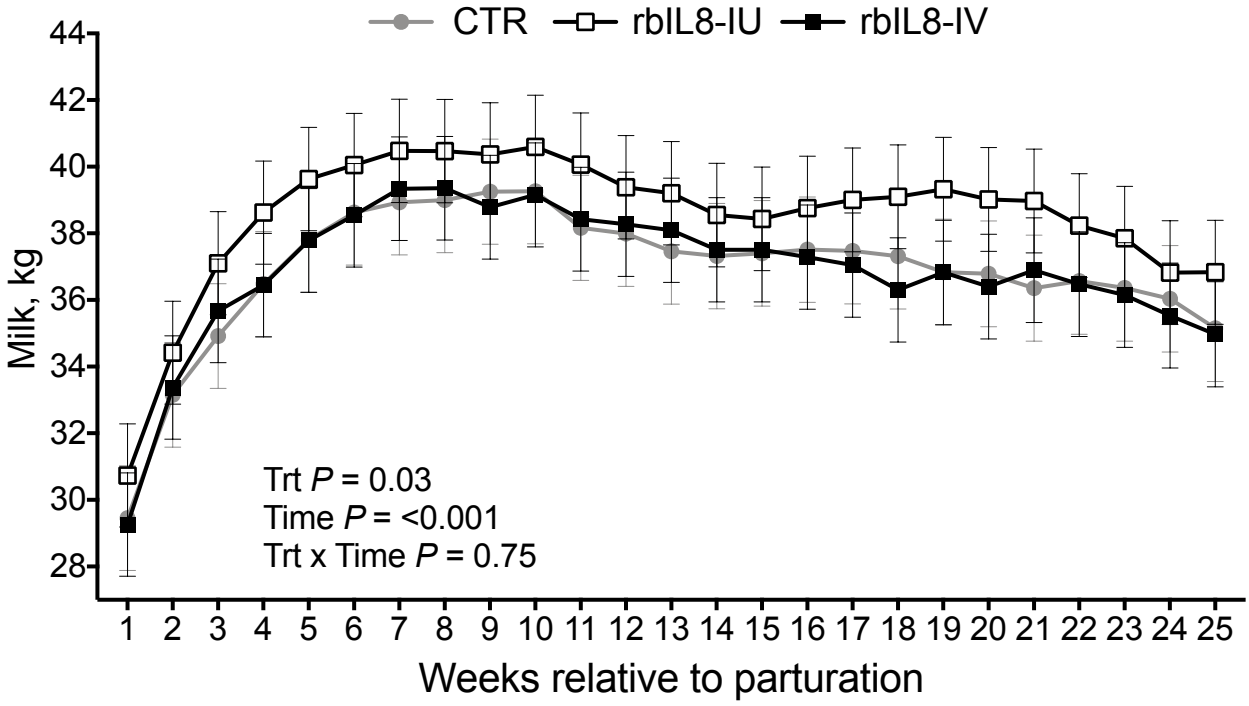


Figure 4.2. Dry matter intake, ECM, FCM, and energy balance (EBAL) for the first 4 weeks of lactation of cows treated with recombinant bovine interleukin-8 intrauterine (rbIL8-IU, n = 70), intravenously (rbIL8-IV, n = 70), and controls (CTR, n = 70). Results are presented as LSM ± SEM. **P* <0.05 (CTR vs IU-IL8).

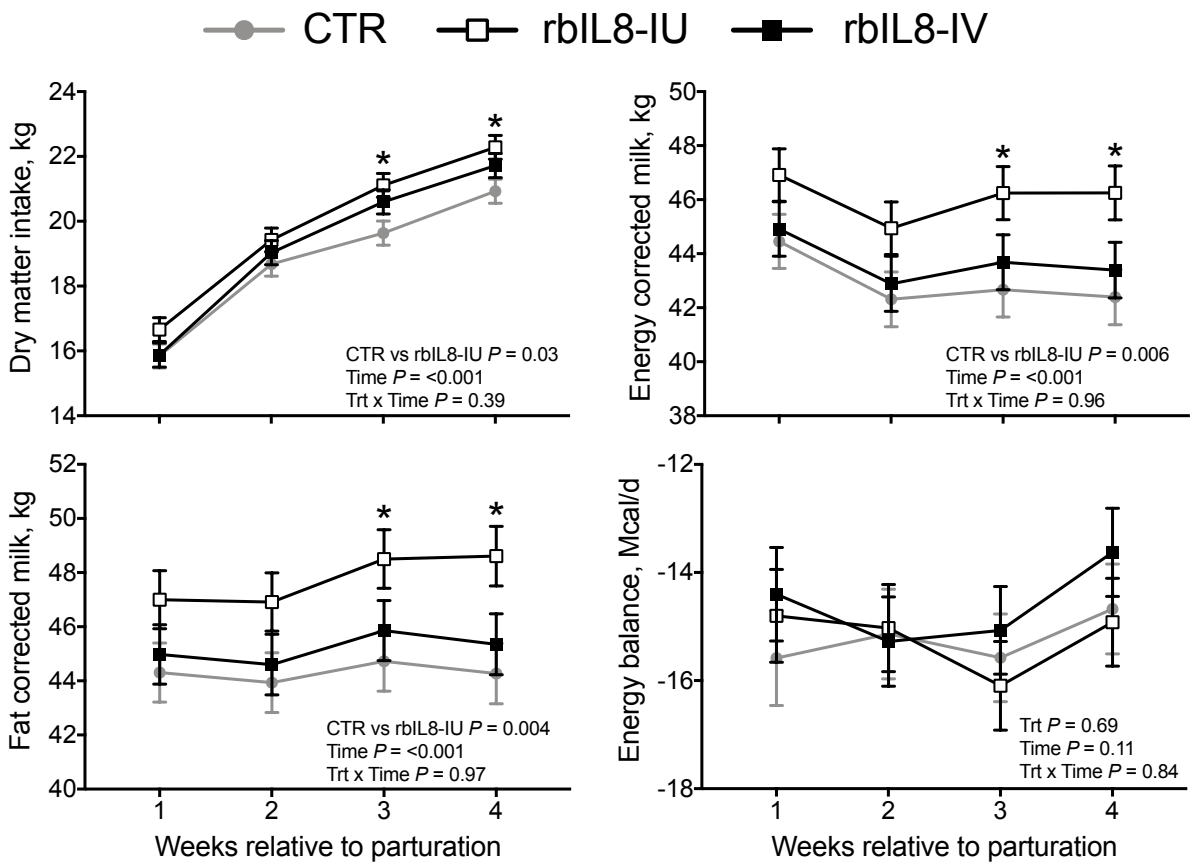


Figure 4.3. Plasma concentrations of insulin, glucose, fatty acids, and BHB for the first 4 weeks of lactation of cows treated with recombinant bovine interleukin-8 intrauterine (rbIL8-IU, n = 70), intravenously (rbIL8-IV, n = 70), and controls (CTR, n = 70). Results are presented as LSM ± SEM.

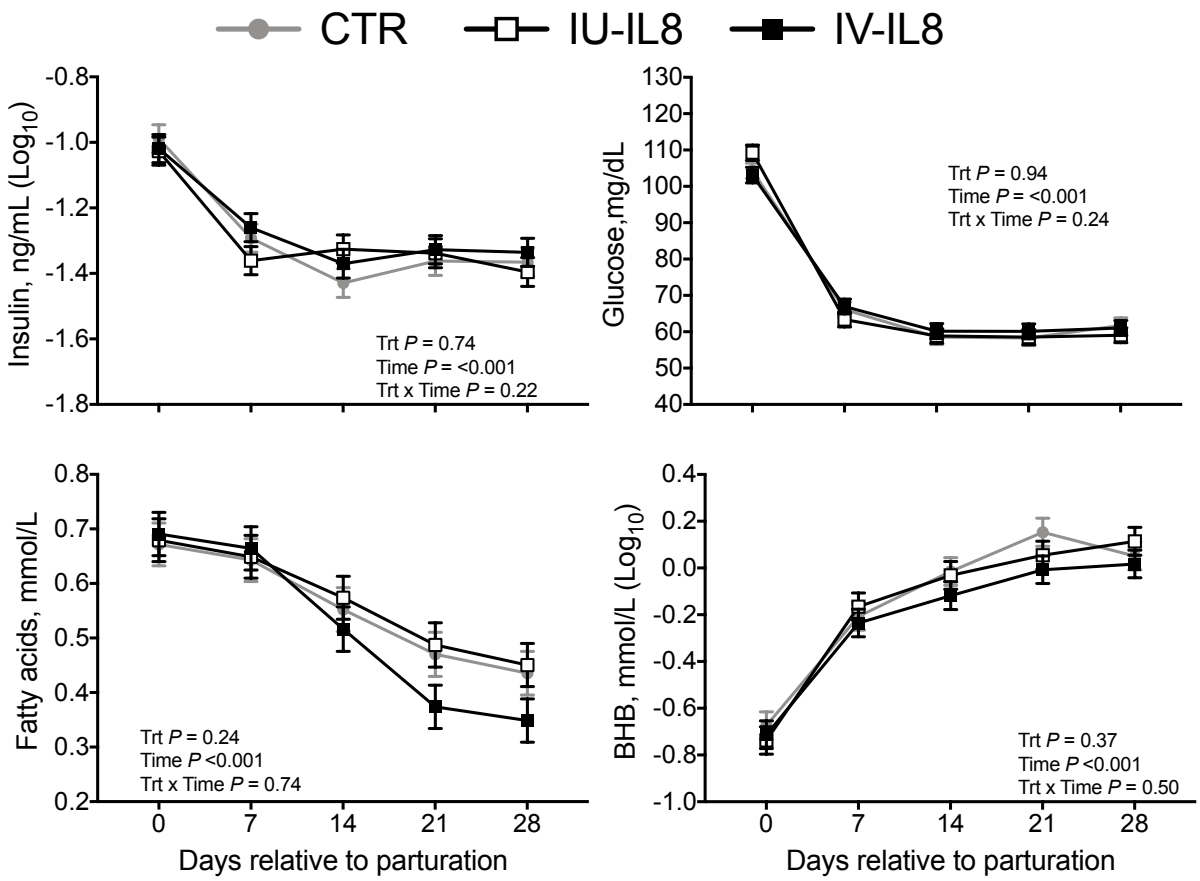


Figure 4.4. Plasma concentrations of growth hormone (GH) and insulin-like growth factor-I (IGF-I) at 0, 7, and 14 d relative to parturition of cows treated with recombinant bovine interleukin-8 intrauterine (rbIL8-IU, n = 70), intravenously (rbIL8-IV, n = 70), and controls (CTR, n = 70). Results are presented as LSM ± SEM.

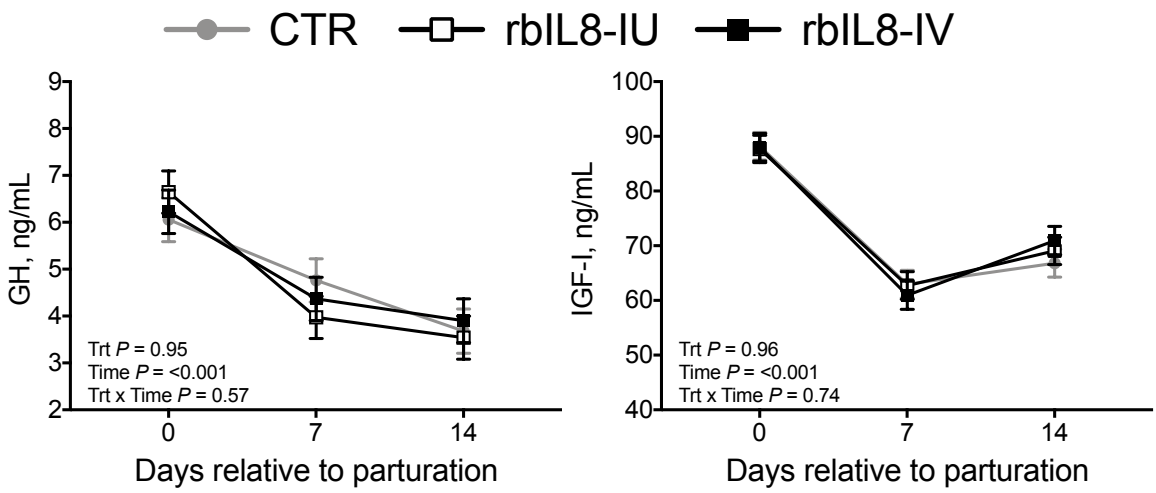


Figure 4.5. Body weight and body condition score loss during the first 90 days of lactation of cows treated with recombinant bovine interleukin-8 intrauterine (rbIL8-IU, n = 70), intravenously (rbIL8-IV, n = 70), and controls (CTR, n = 70). Results are presented as LSM ± SEM.

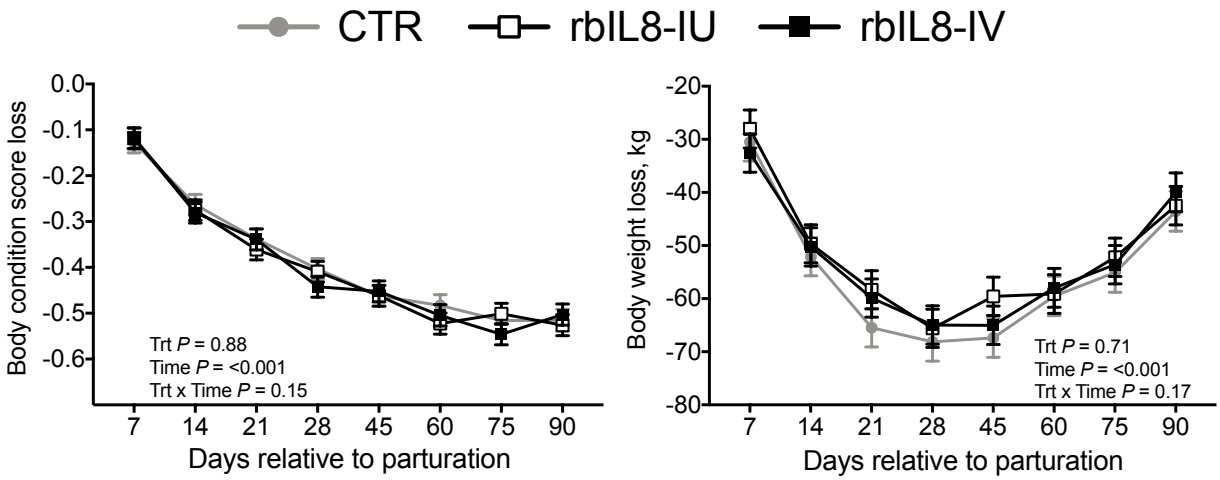


Figure 4.6. Liver triglyceride (TG) levels, liver cell proliferation and apoptosis, the ratio liver cell apoptosis to cell proliferation, and fatty liver incidence (based on TG levels analysis) of cows treated with recombinant bovine interleukin-8 intrauterine (rbIL8-IU, n = 20), intravenously (rbIL8-IV, n = 20), and controls (CTR, n = 20). Liver biopsies were obtained 14 d after treatment. Fatty liver was categorized based on the percentage of liver TG [normal liver (< 1% of liver TG), mild (1-5 % of liver TG), moderate (5-10% of liver TG), and severe fatty liver (> 10% of liver TG); no cows were found with normal or severe fatty liver]. Results are presented as LSM ± SEM for continuous data and as percentage incidence for categorical data.

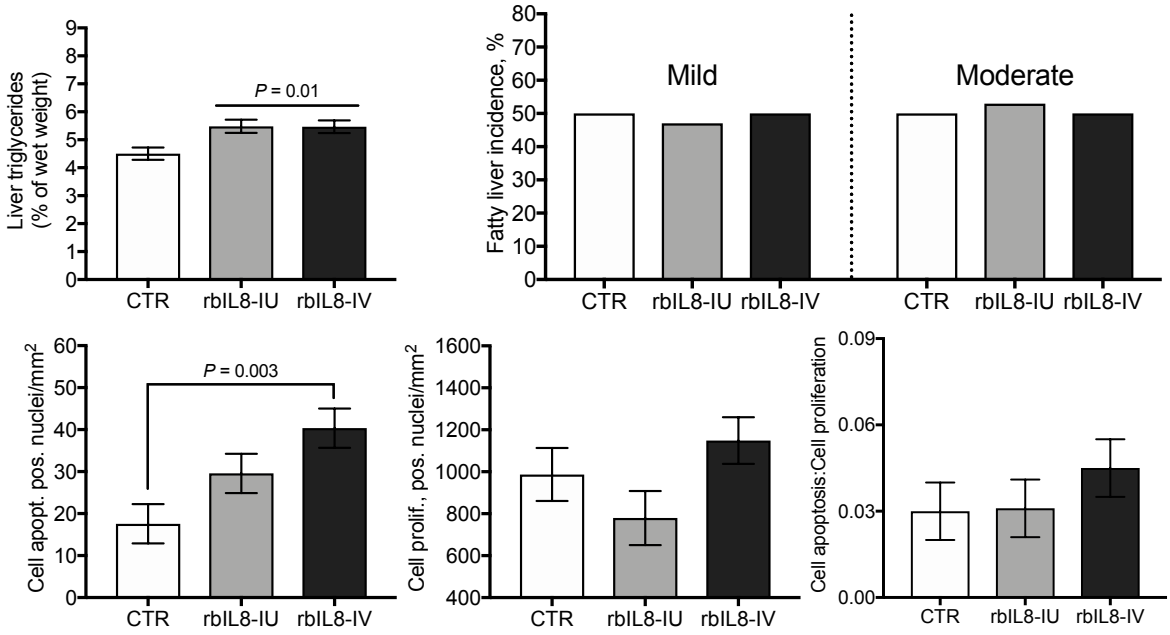


Figure 4.7. Glucose and insulin responses to intravenous glucose tolerance test (IVGTT; panel A) and intravenous insulin challenge test (IVICT; panel B) of cows treated with recombinant bovine interleukin-8 intrauterine (rbIL8-IU, n = 20), intravenously (rbIL8-IV, n = 20), and controls (CTR, n = 20). Intravenous GTT and IVICT were performed on DIM 10 and 11, respectively. White bars, light gray bars, and dark gray bars represent the area under the curve [AUC; mg/dL (glucose), ng/mL (insulin), and mmol/L (fatty acids) per 120 min] of CTR, rbIL8-IU, and rbIL8-IV cows, respectively. * $P \leq 0.05$. Results are presented at LSM ± SEM.

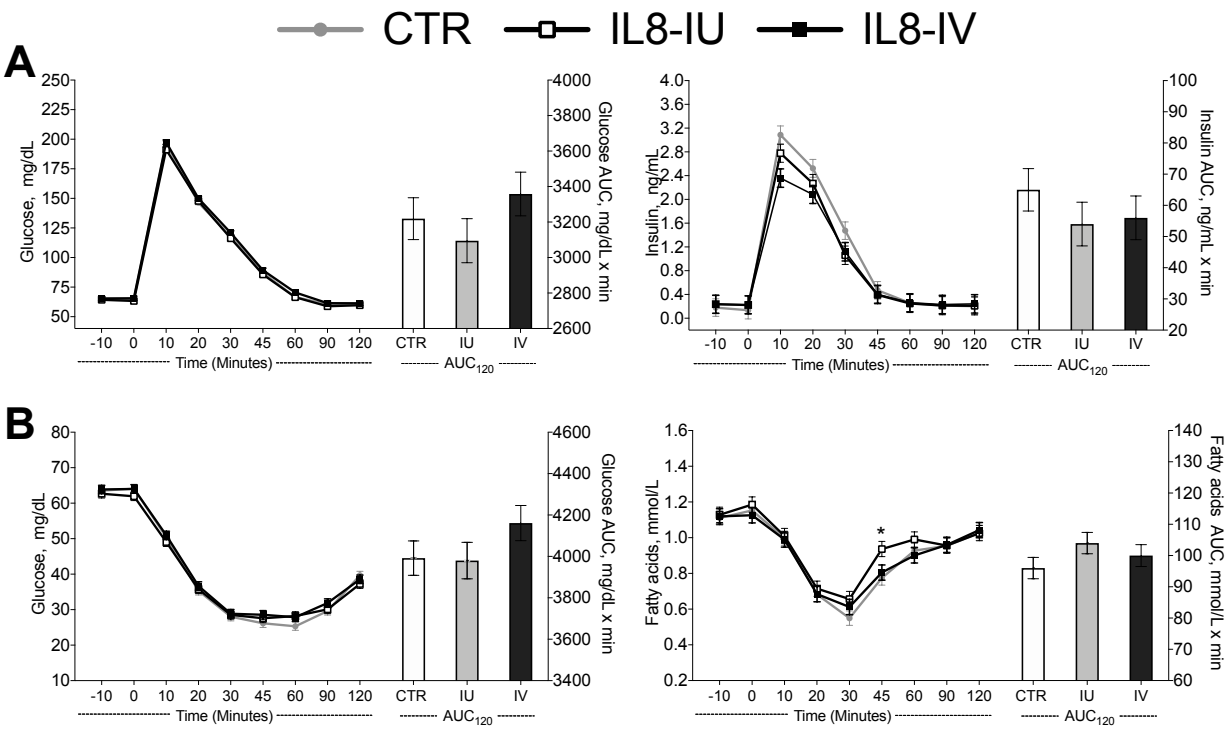
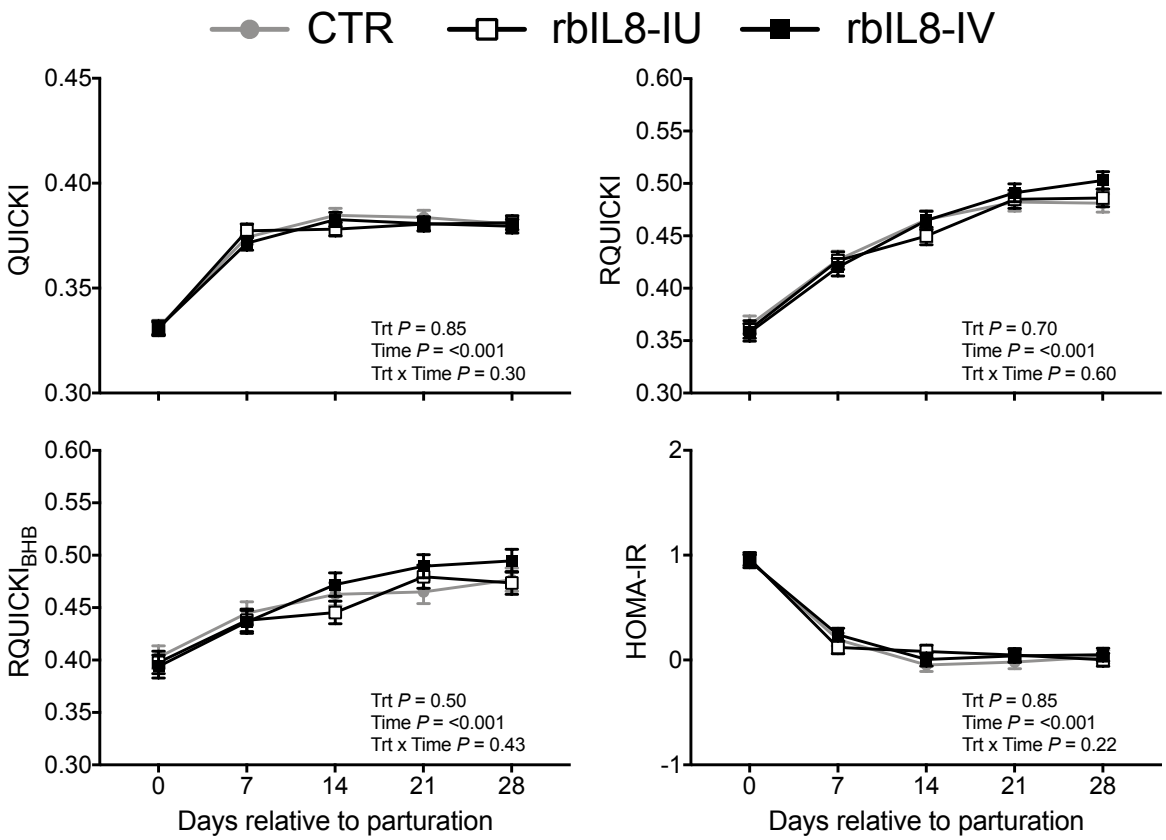
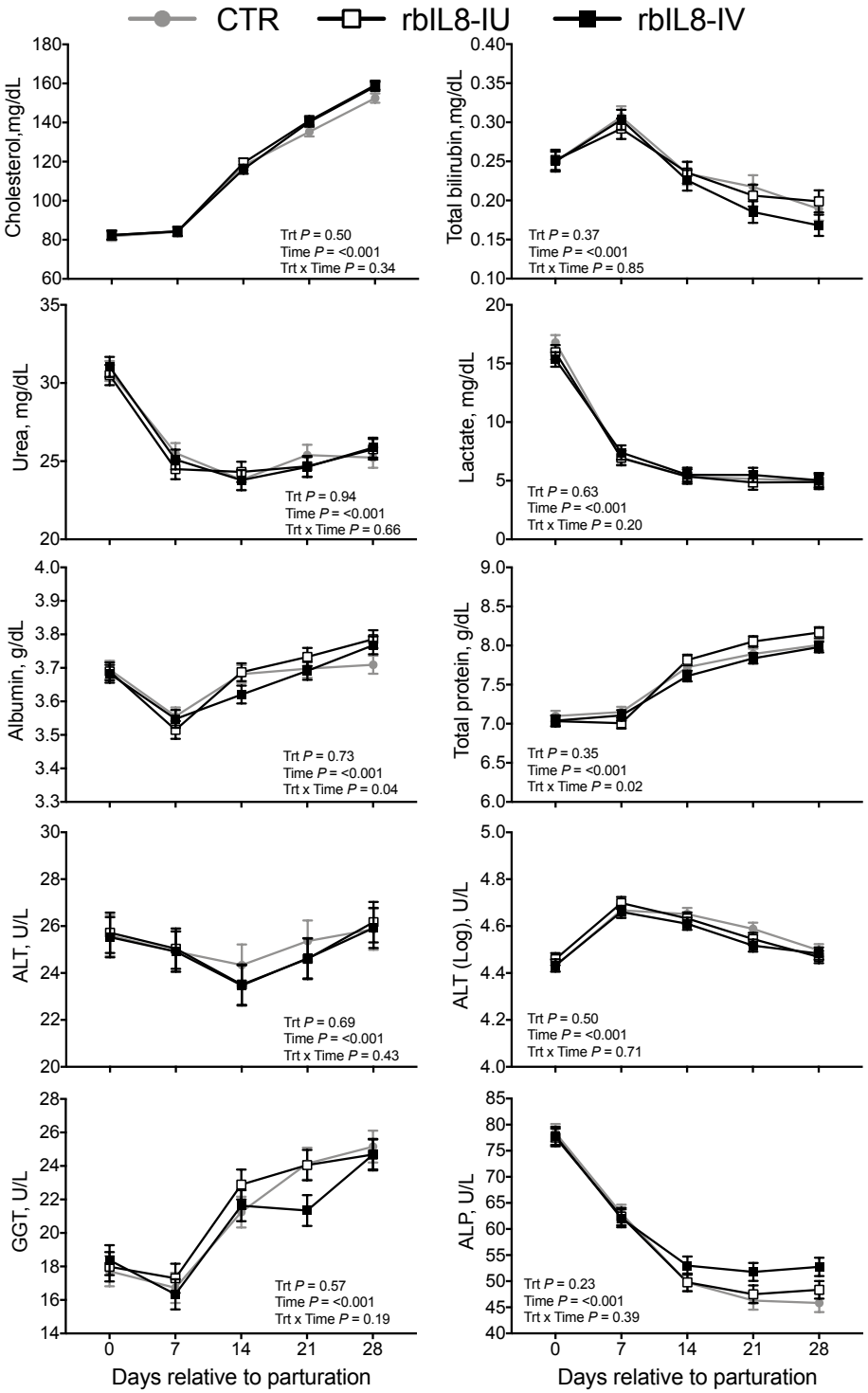


Figure 4.8. Insulin sensitivity indexes for the first 4 weeks of lactation of cows treated with recombinant bovine interleukin-8 intrauterine (rbIL8-IU, n = 70), intravenously (rbIL8-IV, n = 70), and controls (CTR, n = 70). Quantitative insulin sensitivity check index (QUICKI) = {1/[log insulin (μU/mL) + log glucose (mg/dL)]}; revised QUICKI (RQUICKI) = {1/[log glucose (mg/dL) + log insulin (μU/mL) + log fatty acids (mmol/L)]}; revised QUICKI including BHB ($RQUICKI_{BHB}$) = {1/[log glucose (mg/dL) + log insulin (μU/mL) + log fatty acids (mmol/L) + log BHB (mmol/L)]}; and the homeostatic model assessment of insulin resistance (HOMA-IR) = {[glucose (mmol/L) × insulin (μU/mL)]/22.5}. Results are presented as LSM ± SEM.

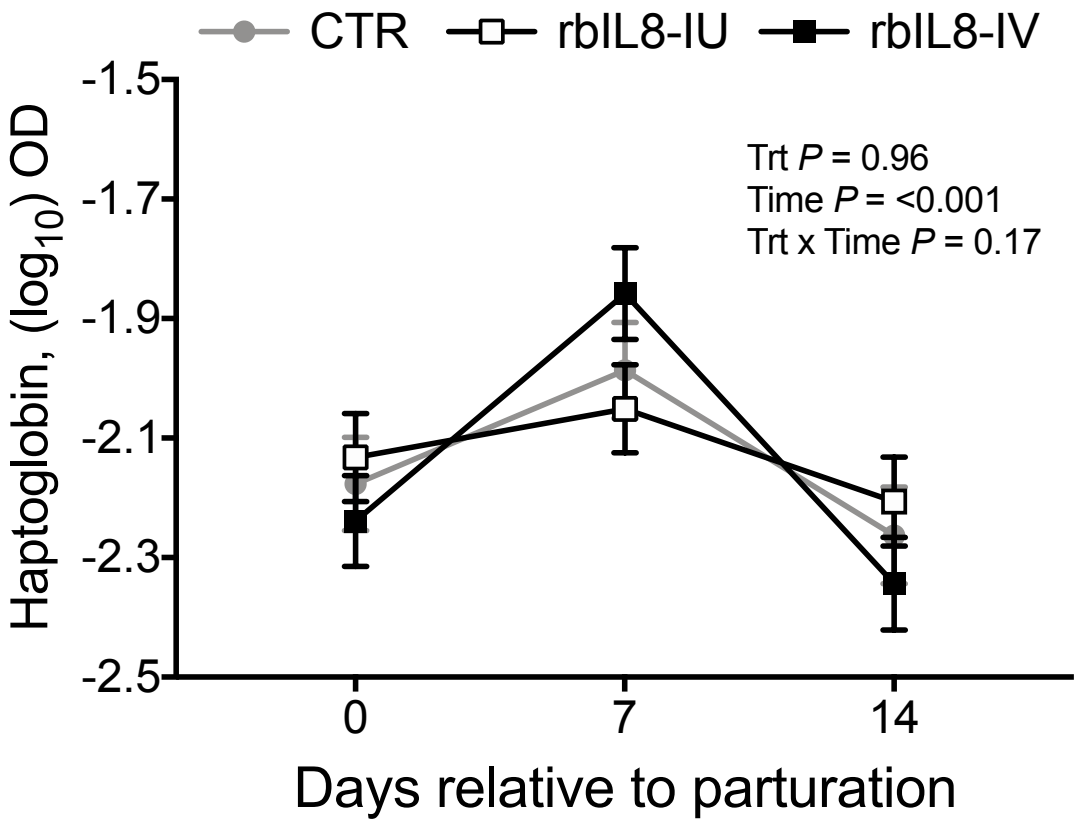


SUPPLEMENTARY INFORMATION

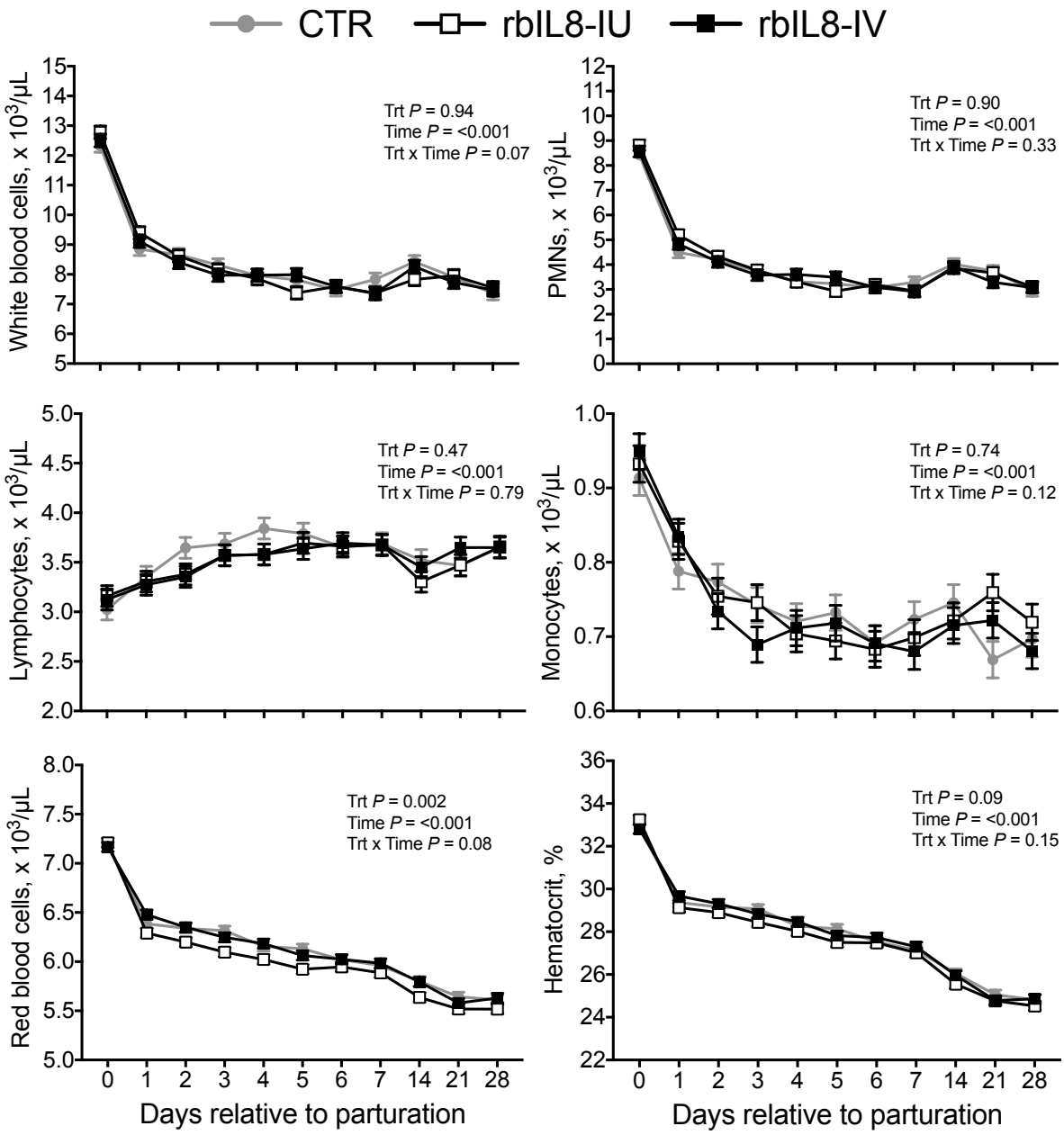
Supplementary Figure 4.1. Metabolites and liver enzymes concentrations for the first 4 weeks of lactation of cows treated with recombinant bovine interleukin-8 intrauterine (rbIL8-IU, n = 70), intravenously (rbIL8-IV, n = 70), and controls (CTR, n = 70). Results are presented as LSM ± SEM.



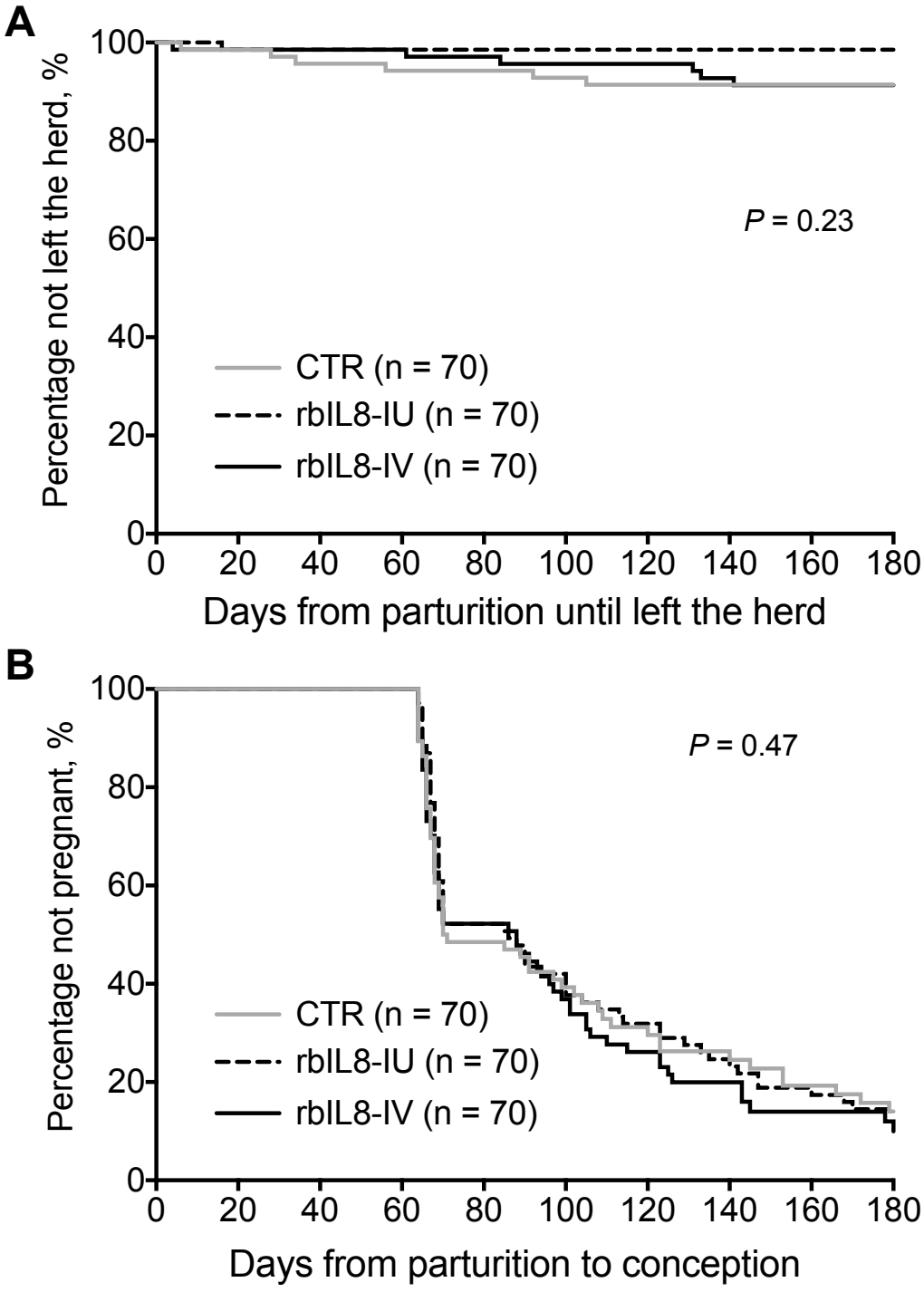
Supplementary Figure 4.2. Plasma haptoglobin (optical density units) concentrations at 0, 7, and 14 d relative to parturition of cows treated with recombinant bovine interleukin-8 intrauterine (rbIL8-IU, n = 70), intravenously (rbIL8-IV, n = 70), and controls (CTR, n = 70). Results are presented as LSM ± SEM.



Supplementary Figure 4.3. Hemogram parameters for the first 28 days postpartum of cows treated with recombinant bovine interleukin-8 intrauterine (rbIL8-IU, n = 70), intravenously (rbIL8-IV, n = 70), and controls (CTR, n = 70). Results are presented as LSM ± SEM.



Supplementary Figure 4.4. **(**A**)** Kaplan-Meier survival curves for calving to left the herd median days of cows treated recombinant bovine interleukin-8 intrauterine (rbIL8-IU), intravenously (rbIL8-IV), and controls (CTR). No differences ($P = 0.23$) were observed between PEG and CTR groups (rbIL8-IU, HR: 0.17, 95% CI: 0.02 to 1.47; rbIL8-IV, HR: 1.06, 95% CI: 0.33 to 3.36). **(**B**)** Kaplan-Meier survival curves for calving to conception interval median days of rbIL8-IU, rbIL8-IV, and CTR cows. No differences ($P = 0.47$) were observed between treatment groups (rbIL8-IU, HR: 0.79, 95% CI: 0.54 to 1.63; rbIL8-IV, HR: 0.94, 95% CI: 0.65 to 1.38).



DISCUSSION

This study was conducted to explore underlying mechanisms by which rbIL-8 treatment increases milk production in Holstein cows. In line with our previous studies (Zinicola et al., 2018), the IU administration of rbIL-8 within 12 h of parturition significantly increased milk, FCM, and ECM yields in Holstein cows with no changes in BCS. Furthermore, intravenous administration of rbIL-8 did not suppress DMI or milk production and was not associated with any negative side effects. Moreover, BW and EBAL were not affected by rbIL-8 treatment. Interestingly, the administration of rbIL8-IU resulted in higher DMI during the first 4 wk postpartum. Treatment with rbIL8-IU reduced the incidence of HYK and overall diseases when compared with CTR while not altering early lactation metabolism; none of the hormones and metabolites herein evaluated were affected by treatment. In addition, peripheral tissue insulin sensitivity was not altered by rbIL-8 treatment. Therefore, it is likely that the observed long-term production responses following rbIL8-IU treatment are as a result of increased DMI likely derived from improved overall health. In support to this notion, the ratio of milk and ECM to DMI were similar among treatment groups.

We have previously shown that cows that received a single IU administration of rbIL-8 shortly after parturition produced more milk during the first 6 months of lactation with no changes on BCS (Zinicola et al. 2018a). Because cows treated with rbIL-8 had similar BCS loss during the first 35 DIM compared with controls, it is plausible to propose that rbIL-8 treated cows had increased DMI during the same period. Unfortunately, in that study, it was not possible to measure DMI. In the present study, we confirmed that rbIL-8 treated cows produced more milk, while no differences in BCS and body weight loss were observed from placebo treated animals. Importantly, we demonstrated for the first time that a single administration of rbIL8-IU in Holstein cows within 12 h of parturition was associated with increased DMI during the first 30 DIM. To the best our knowledge this is the first report to demonstrate an effect of rbIL-8 treatment on DMI in an animal model. It is also unforeseen, since other pro-inflammatory

cytokines have been repeatedly associated with appetite suppression and in some reports with a drastic reduction in milk production (Langhans and Hrupka, 1999; Yuan et al., 2013).

A recent study has evaluated the effects of recombinant bovine tumor necrosis-α (**rbTNF-α**) treatment in dairy cows during the early lactation on dry matter intake, metabolism, and milk production (Yuan et al., 2013). Cows that received rbTNF-α had significantly lower DMI, lower water intake, and produced significantly less milk when compared with controls. Insulin is one of the most important anorexigenic hormone acting on hypothalamus (Plum et al., 2005). In the study conducted by Yuan et al. (2013), rbTNF-α treatment did not alter the concentration of plasma insulin and dramatic changes on metabolism were not observed. These results reinforced the well-known potent anorexigenic effect of rbTNF-α acting directly in the hypothalamus (Romanatto et al., 2007). Moreover, rbTNF-α treatment increased plasma haptoglobin concentrations (Yuan et al., 2013). Interleukin-8 is also a potent pro-inflammatory chemokine capable of activating and attracting neutrophils, but the body of knowledge generated by our group over several different controlled trials (Bicalho et al., 2018; Zinicola et al., 2018a), indicate that the detrimental effects on appetite, metabolism, and health are not observed after a one-time IV or IU administration.

Lipopolysaccharide (**LPS**) challenge models have been used in many different animal species to advance our understanding about the interaction of immunological responses and metabolism (Spurlock, 1997; Kvidera et al., 2017). Specifically, LPS challenge is associated with an increase in white blood cells, fever, an increase in acute-phase proteins, elevated inflammatory mediators, depressed appetite, and dramatic changes on metabolism (Mulligan et al., 2012; Dillingh et al., 2014; Kvidera et al., 2017). Recently, Kvidera et al., (2017) studied the whole-body glucose utilization following a single LPS bolus challenge while exploring the metabolic and production changes in mid-lactation Holstein cows. They observed that LPS challenge resulted in a quick increase in circulating glucose concentration, likely as a result of glycogenolysis, followed by a period of decreased glucose concentration. Because of LPS challenge, a dramatic and sustained increase in plasma insulin (6-fold increase) was reported.

The increase in plasma insulin observed in that study, likely redirected energy resources towards insulin dependent tissues, decreased gluconeogenesis, suppressed appetite, and halted lipolysis, which would explain the resulting dramatic decrease in DMI and milk production. That metabolic milieu, which characterizes the metabolic changes of an animal with an activated immune system, was not observed in cows treated with rbIL8-IU or rbIL8-IV.

We are not aware of any possible direct effect or mechanism that could link IL-8 with a central regulation of feed intake. On the contrary, a study showed that intracerebroventricular administration of recombinant human IL-8 (rhIL-8) in rats was associated with reduced food intake (Plata-Salaman and Borkoski, 1993). In that study, however, the peripheral administration of rhIL-8 in doses similar to those administered centrally did not alter food intake. Herein, the administration of rbIL8-IV was not associated with DMI. Thus, we suggest that IL-8 does not increase DMI by stimulating the feeding centers of the brain. Uterine infections are associated with depressed DMI during the pre and postpartum period (Hammon et al., 2006; Huzzey et al., 2007). Uterine diseases are highly associated with the presence of *E. coli* and its LPS in lochia during the very early postpartum (Dohmen et al., 2000; Bicalho et al., 2012). We have demonstrated that rbIL-8 is effective to attract neutrophils to the reproductive tract in Holstein animals (Bicalho et al., 2018). Moreover, rbIL8-IU treatment significantly reduced the incidence of puerperal metritis in multiparous cows (Zinicola et al., 2018a). The immune system energy expenditure is extremely high in dairy cows facing endotoxin challenges such us uterine bacterial infections (Kvidera et al., 2017). Thus, it is reasonable to surmise that an improved local immune response in the uterus caused by rbIL8-IU might have reduced the endotoxin challenges for these cows and triggered the increased DMI that drove the differences in milk production reported in the current study. However, more studies are needed to elucidate the mechanism by which rbIL8-IU treatment increases DMI in lactating cows.

An increase in hematocrit levels is characteristic of dehydration. Herein, we observed that rbIL8-IU cows tended to have lower hematocrit levels when compared with CTR cows. We also observed that cows treated with rbIL8-IU had significantly lower red blood cell numbers than

CTR cows. In a dehydration status, the volume of fluid in the blood drops, thus the count of red blood cells rises. Therefore, it is possible that water intake may have also increased in the rbIL8-IU treated cows.

One of the main hypotheses by which rbIL-8 treatment increases milk production was through stimulation of insulin resistance. A study in the human literature demonstrated that IL-8 promotes insulin resistance via the inhibition of AKT activation through ERK and/or p38 MAPK pathways (Kobashi et al., 2009). Moreover, we have demonstrated that repeated systemic administration of rbIL-8 in male Holstein calves induced a long-lasting state of insulin resistance (Zinicola et al., 2018). In contrast with our hypothesis, in the present study rbIL-8 treatment did not affect peripheral tissue insulin sensitivity, as measured by IVGTT, IVICT, and insulin sensitivity indexes. Thus, we can conclude that promoting insulin resistance does not seems to be the mechanism by which rbIL8-IU treatment increases milk yield in cows.

We also observed that rbIL8-IU treatment reduced the incidence of postpartum HYK, which also agree with our previous findings (Zinicola et al., 2018). We have proposed that the underlying mechanism by which rbIL8-IU reduces the incidence of HYK could be increased liver cell proliferation and/or reduced apoptosis (Colletti et al., 1998; Osawa et al., 2002). The increased number of cells available to oxidize fatty acids could lower BHB production and lower TG accumulation within hepatocytes (Zinicola et al., 2018a). However, in the current study rbIL8-IU treatment did not alter cell turnover indices, as the ratio of liver cell apoptosis to cell proliferation (indicative of cell production) was similar among treatments. Thus, the observed increased DMI in cows treated with rbIL8-IU leads us to suggest that lower incidence of HYK following rbIL8-IU administration may be as a consequence of higher DMI. Along this line, we also suggest that the observed reduced incidence of diseases in cows treated with rbIL8-IU might be as a result of increased DMI. However, it is unclear if the increased DMI resulted in improved health or if the improved health resulted in higher DMI.

It is surprising that cows treated with rbIL8-IU and rbIL8-IV had higher levels of liver TG when no effects on rbIL-8 treatment on peripheral tissue insulin sensitivity, plasma

concentrations of fatty acids, BHB, and liver cell production were found. We do not have a clear explanation for this observation. In lactating cows, the accumulation of TG in the liver begin near to the central hepatic vein and progresses toward the midzonal region of the liver (Veenhuizen et al., 1991). Since the collection of liver tissue sample is a blind procedure, it is possible that liver samples could have been taken from different areas of the liver, and thus affecting the interpretation of the results. Therefore, the increased liver TG concentration observed as a consequence of rbIL-8 treatment could have been a type I error. Further investigations are needed to confirm whether increased liver TG levels should be expected in cows treated with rbIL-8. Besides the observed significant increase of liver TG levels in treated cows, it is important to highlight that when cows were categorized into normal, mild, moderate, and severe fatty liver based on TG concentration (Bobe et al., 2004), no differences were observed between treatment groups. The incidence of fatty liver is associated with the incidence of ketosis (Veenhuizen et al., 1991). In the present study, despite the observed increase on liver TG levels in cows treated with rbIL-8, the incidence of CK was not affected by treatment. Indeed, a numerical reduction of CK incidence was observed in cows treated with rbIL-8. Additionally, no differences were detected on hepatic enzymes and others indirect markers of liver functionality/damage between treatment groups.

Cytokines are known to alter the GH signaling via SOCS proteins production (Rico-Bautista et al., 2006). In a previous study, we suggested that rbIL-8 treatment might favors the un-coupling of the GH/IGF-1 axis that postpartum cows experience during early lactation as a homeorhetic shift to support milk production (Zinicola et al., 2018). In contrast with this hypothesis, we have shown that Holstein calves treated with rbIL-8 have similar GH and IGF-1 concentrations than placebo treated calves (Zinicola et al., 2018). In the present study, plasma GH and IGF-1 levels were not affected by treatment. Therefore, it is reasonable to suggest that rbIL-8 treatment does not alter the GH and IGF-1 axis in dairy cows.

Moreover, in a previous study we also hypothesized that the observed higher milk yield following rbIL-8 treatment might be a result of increased mammary cell number. The activation

of NF-κB during mammary gland development is critical for mammary cell differentiation (Brantley et al., 2001; Cao et al., 2001). Interleukin-8 is known as an activator of NF-κB (Bendre et al., 2003), which will up-regulate cycling D1 and therefore promote cell differentiation (Srivastava et al., 2003). Considering the observed increased DMI in cows treated with rbIL8-IU we surmise that it is unlikely that IL-8 would increase milk production by increasing mammary gland cell proliferation. However, further studies are needed to assess this possibility.

In agreement with our previous study, plasma haptoglobin concentrations and RT were not affected by rbIL-8 treatment (Zinicola et al., 2018). In addition, we did not observe changes on blood cell numbers between treatment groups. These results suggest that the administration of rbIL-8 in lactating cows does not alter systemic inflammation.

Long-term milk production responses were not observed in cows treated with rbIL8-IV. Cows treated with rbIL8-IV had similar DMI when compared with CTR cows. Therefore, considering that rbIL8-IU cows produced more milk and had significantly higher DMI than CTR cows, the lack of effect of rbIL8-IV treatment on milk production is not surprising. These results also provide further support to the notion that the observed increase in milk production and DMI as well as improved health following rbIL8-IU are a consequence of a local effect in the uterus.

CONCLUSIONS

This study was conducted to explore underlying mechanisms by which rbIL-8 treatment increases milk production in Holstein cows. We observed that cows treated with rbIL8-IU produced more milk, FCM, and ECM yields than CTR cows, with no changes on BCS, BW, and EBAL. In contrast with our hypotheses, no differences were observed in metabolism in response to rbIL-8 treatment. Moreover, rbIL8-IU treatment significantly increased DMI during the first 4 wk postpartum. The administration of rbIL8-IU also reduced the incidence of HYK and improved overall postpartum health. We conclude that the long-term effects of rbIL8-IU treatment on milk production and health may be mainly because of increased DMI. Further research is necessary to elucidate the mechanism by which IU administration of rbIL-8 increases

voluntary feed intake in lactating cows. This study supports the use of rbIL-8 administered IU shortly after calving to improve health and production responses in lactating cows.

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CHAPTER 5: Association of peripartum plasma insulin concentration with milk production, colostrum insulin levels, and plasma metabolites of Holstein cows*

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ABSTRACT

The main objective of this study was to assess associations between plasma insulin concentration around parturition and production in Holstein cows. Primiparous and multiparous cows (n = 267) were enrolled. Blood samples were collected within 12 h after parturition (d 0), and on d 3 and 10 after calving. In addition, blood samples were collected 7 d before (-7 d) the expected date of parturition and colostrum samples were collected within 8 h after parturition from a subset of cows to measure insulin concentration (n = 47). All samples were harvested from 0630 to 1100 h and were used to quantify insulin, non-esterified fatty acids (**NEFA**), and β-hydroxybutyrate. The plasma concentrations of insulin on d -7 and 0 were not correlated with insulin levels in colostrum. Cows were grouped according to plasma insulin concentration based on the median as low insulin (**L-INS**) or high insulin (**H-INS**) on d 0 [median = 0.35 ng/mL; range (0.2 to 1.2)], 3 [median = 0.32 ng/mL; range (0.2 to 1.6)], and 10 [median = 0.30 ng/mL; range (0.2 to 0.8)]. We detected that cows in the L-INS group on d 0 (L-INS = 0.57 ± 0.02; H-INS = 0.49 ± 0.02 mmol/L), d 3 (L-INS = 0.56 ± 0.02; H-INS = 0.49 ± 0.02 mmol/L), and d 10 (L-INS = 0.61 ± 0.03; H-INS = 0.55 ± 0.03 mmol/L) had higher NEFA concentrations compared with cows in the H-INS group. Compared with H-INS cows, milk yield was higher for cows classified as L-INS on d 0 (L-INS = 40.75 ± 0.69; H-INS = 38.41 ± 0.64 kg) and d 10 (L-INS = 40.95 ± 0.74; H-INS = 38.66 ± 0.64 kg). Moreover, fat-corrected milk was higher for cows classified as L-INS on d 0 (L-INS = 40.59 ± 2.36; H-INS = 37.73 ± 2.31 kg) and d 10 (L-INS = 41.00 ± 2.42; H-INS = 38.65 ± 2.28 kg) compared with H-INS cows, and energy-corrected milk was higher for L-INS cows compared with H-INS cows regardless of the day (d 0 - L-INS = 44.50 ± 0.70 vs H-INS = 41.67 ± 0.64 kg; d 3 - L-INS = 43.65 ± 0.74 vs H-INS = 40.88 ± 0.72 kg; d 10 - L-INS = 44.09 ± 0.73 vs H-INS = 40.55 ± 0.68 kg). We conclude that low plasma insulin concentration during early lactation is associated with higher milk yield in the long-term.

Keywords: Insulin, milk production, NEFA, BHB

INTRODUCTION

Insulin is an anabolic hormone secreted by β-cells of the islets of Langerhans in the pancreas in response to increased levels of glucose circulating in the blood. Binding of insulin to its cell surface receptor stimulates glucose uptake by cells, decreases gluconeogenesis, glycogenolysis, and lipolysis, increases fat and protein synthesis, and promotes cell division and cell growth (Brockman and Laarveld, 1986). However, in postpartum dairy cows, circulating concentration of insulin is extremely low to prioritize the use of glucose for milk production (Hart, 1983). In addition, it is considered that Holstein cows undergo a transient state of insulin resistance, which might increase the supply of blood glucose for lactose production by the insulin-independent mammary gland (Bell and Bauman, 1997; De Koster and Opsomer, 2013). These major endocrine adaptations are part of the homeorhetic shift that characterizes the lactating dairy cow at the beginning of lactation (Bauman and Currie, 1980; Bell and Bauman, 1997).

Several studies have investigated the effect of insulin administration on milk yield in lactating dairy cows. For instance, Schmidt (1966) showed that repeated injections of a short-acting insulin into primiparous Holstein cows resulted in decreases in milk yield and milk lactose, and increases in milk fat and protein (Schmidt, 1966). In a study by Kronfeld et al. (1963), cows that received repeated high doses of a long-acting insulin had reduced milk yields (Kronfeld et al., 1963). In both studies, milk production was restored to pre-insulin treatment after the cows were infused with exogenous glucose. These findings suggested that the observed reduction in milk yield following insulin administration was due to a reduction in blood glucose concentration owing to insulin's hypoglycemic effect. Nevertheless, a recent study that used a lower dose of long-acting insulin administered twice daily for a period of 10 d, and at different stage of lactation (at around 90 DIM), observed that milk yield did not differ between cows that received insulin and those that did not (Winkelman and Overton, 2013). The concentration of lactose in milk, however, was significantly lower among insulin-treated cows (Winkelman and Overton, 2013).

However, limited evidence exists regarding the associations of periparturient plasma insulin and milk yield of Holstein cows. Thus, our hypothesis was that low plasma insulin levels around calving and during early lactation would be positively associated with milk production. Our primary objective was to explore the association of plasma insulin at different DIM during the immediate postpartum with milk production. We also explored the association between postpartum plasma insulin and NEFA and BHB concentrations.

MATERIAL AND METHODS

Farm and Management

An observational study was conducted in a large commercial dairy farm located in Cayuga County near Ithaca, NY. The farm milked ~3,600 Holstein cows thrice daily in a rotary parlor with integrated milk meters that recorded individual production at every milking (DeLaval, Tumba, Sweden). The cows were housed in free-stall barns, with concrete stalls bedded with composted manure solids. After calving, all cows were offered a TMR of approximately 55% forage (corn silage, haylage, and wheat straw) and 45% concentrate (corn meal, soybean meal, canola, cottonseed, and citrus pulp) on a dry matter basis. The TMR was fed at approximately 0800 h and feed was pushed up 6-8 times a day. The diet was formulated to meet or exceed the NRC nutrient requirements for lactating Holstein cows weighing 650 kg and producing 45 kg of 3.5% FCM (NRC, 2001). As cows demonstrated signs of calving, they were moved to individual maternity pens for delivery, where trained farm personnel assisted with parturition as needed. Calves were removed from their dams immediately after birth. The Presynch-Ovysnch protocol, in combination with estrus detection and artificial insemination, was used for the first service (Pursley et al., 1995; Moreira et al., 2001). A combination of heat detection and the Resynch (Fricke et al., 2003) protocol was used to inseminate cows after the first service. A voluntary waiting period of 50 days was used. Estrus was detected based only on electronic activity sensors (Alpro, DeLaval, MO) worn around the neck.

Study Design and Animal Sampling

In total, 267 Holstein dairy cows (primiparous, n = 98; multiparous, n = 169) were enrolled in the study from October to December 2015. Blood samples, collected from 0630 to 1100 h, were obtained from all study cows from the coccygeal vein or artery using 10-mL vacutainer K2 EDTA blood collection tubes (BD Vacutainer, Franking Lakes, NJ) within 12 h (d 0), and at 3 (2 to 5 d) and 10 (8 to 11 d) d relative to parturition. After collection, samples were placed on ice, and plasma was obtained within 3 h by centrifugation at 2,000 × *g* for 15 minutes at 4 °C, and frozen at -20 °C. A total of 18 blood samples were not collected on d 3 and 1 sample was not collected on d 7. Additionally, blood and colostrum samples from a subset cows (primiparous, n = 20; multiparous, n = 27) were collected at -7 ± 3 d (blood) and within 8 h after calving (colostrum). Colostrum was collected by trained farm personnel following recommendations of the research group. Briefly, teats were cleaned and disinfected, the initial 2 streams were discarded, and approximately 10 mL of colostrum from each quarter was collected into a sterile 50 mL falcon tube without preservative (VWR, Chicago, IL). Colostrum samples were transported on ice to the laboratory, homogenized by vortex-mixing, and aliquots of 2 mL were centrifuged at 10,000 × *g* for 15 minutes at 4 °C, and supernatant was stored at -20 °C for further analysis. Body condition scores (**BCS**) were determined at 0 and at 35 (32 to 38 d) DIM using a five-scale, quarter-point system as previously described (Edmonson et al., 1989). Weekly milk yield average was recorded for 12 weeks after calving. Four cows were culled on wk 7, 3 on wk 9, and 2 on wk 11.

Blood and Colostrum Analysis

Insulin concentration was determined using a commercial ELISA kit for bovines (Bovine Insulin ELISA, ALPCO®, Salem, NH). Plasma concentrations of NEFA (NEFA-C® kit; Wako Pure Chemical Industries, Richmond, VA) and BHB (Williamson and Mellanby, 1974; Sigma-Aldrich, St. Louis, MO) were determined by colorimetric methods. The intra-assay and inter-assay coefficients of variation for the assays were <8.5% and <10%, respectively.

Statistical Analysis

Sample size calculation was conducted using JMP PRO (version 12; SAS Institute Inc., Cary, NC). Considering an average standard deviation of 3.7 kg/d for milk yield (Douglas et al., 2006; Smith et al., 2009; Greco et al., 2015) and assuming a type I error rate of 5% and a power of 80%, a total of 110 cows per group were deemed necessary to detect a significant difference of 2 kg/d between groups. However, an additional ~20% of cows were enrolled in the study to compensate for cows removed from the herd during the study period beyond the control of the investigators.

Descriptive statistical analysis was performed using the UNIVARIATE procedure in SAS (version 9.4; SAS/STAT, SAS Institute Inc., Cary, NC). Cows were classified into high plasma insulin (H-INS) or low plasma insulin (L-INS) based on the median plasma insulin levels at d 0, 3, and 10 relative to parturition. Continuous variables evaluated over time [insulin, NEFA, BHB, weekly average milk yield, monthly test day milk (**TDM**), 3.5% FCM, ECM, percent fat, and percent protein] were analyzed using general linear repeated measures mixed models with the MIXED procedure of SAS. To account appropriately for within-cow correlation, the error term was modeled by imposing a first-order autoregressive covariance structure for all statistical models. Fixed effects of group (H-INS/L-INS at d 0, 3 or 10), time, parity, BCS at calving, dystocia, twins, stillbirth, and the two-way interaction terms group by parity, and group by time were offered to the models. Tukey's honest significance test for multiple comparisons was used. Data are reported as least squares mean (**LSM**) ± standard error of the mean. Differences in sire predicted transmitting ability for milk yield between cows classified as low or high insulin concentration at different time points was evaluated using the GLIMMIX procedure of SAS. The relationship between plasma insulin at d -7 and 0 after calving and the concentration of insulin in colostrum was analyzed with the Spearman correlation test using the multivariate method of JMP PRO.

For all models, variables and their respective interaction terms were retained in the model when $P \leq 0.15$. Significance was considered when $P \leq 0.05$ or a tendency if $0.05 < P \leq 0.10$.

RESULTS

Descriptive Data

The median plasma insulin concentration on d 0 was 0.35 ng/mL [range (0.20 to 1.20)] and 133 cows (primiparous, n = 29; multiparous, n = 104) were identified as L-INS [range (0.20 to 0.35)], whereas 134 cows (primiparous, n = 69; multiparous, n = 65) were identified as H-INS [range (0.36 to 1.20)]. The median plasma insulin concentration on d 3 was 0.32 ng/mL [range (0.20 to 1.60)] and 125 cows (primiparous, n = 37; multiparous, n = 88) were identified as L-INS [range (0.20 to 0.32)], whereas 124 cows (primiparous, n = 57; multiparous, n = 67) were identified as H-INS [range (0.33 to 1.60)]. Lastly, the median plasma insulin concentration on d 10 was 0.30 ng/mL [range (0.20 to 0.80)] and 133 cows (primiparous, n = 33; multiparous, n = 100) were identified as L-INS [range (0.20 to 0.30)], whereas 133 cows (primiparous, n = 64; multiparous, n = 69) were identified as H-INS [range (0.31 to 0.80)].

Supplementary table 5.1 illustrates the frequency distribution of each insulin classification group (L-INS or H-INS) for sample collection days 0, 3, and 10.

Colostrum and Plasma Insulin levels around Parturition

Figure 5.1 illustrates the insulin plasma concentrations at d -7, 0, 3, and 10 relative to parturition, and the colostrum insulin levels by parity from a subset of cows (n = 47). Primiparous cows had lower ($P < 0.05$) plasma insulin levels on d 0, 3, and 10 compared with -7 d (Figure 5.1A). Multiparous cows had lower ($P < 0.05$) plasma insulin levels on d 3 and 10 after calving compared with d -7 (Figure 5.1B). The mean ± SEM of colostrum insulin concentration for primiparous cows was 106.74 ± 14.9 ng/mL (Figure 5.1A) and for multiparous cows it was 53.99 ± 6.12 ng/mL (Figure 5.1B).

Relationships between Colostrum Insulin and Plasma Insulin

The relationships between insulin concentration in colostrum and plasma at d -7 and 0 relative to parturition are depicted in Figure 5.2. Colostrum insulin concentration was not associated with plasma insulin levels, irrespective of the day. The correlation between insulin level in plasma versus that in colostrum on d -7 and 0 relative to parturition was r = -0.08 (P = 0.57) (Figure 5.2A) and r = 0.12 (P = 0.42) (Figure 5.2B), respectively.

Association between Insulin classification and NEFA and BHB Concentrations

Cows classified into L-INS or H-INS at day 0

Plasma concentrations of insulin, NEFA, and BHB are illustrated in Figure 5.3A. Cows grouped as L-INS had lower (P < 0.001) plasma insulin when compared with H-INS cows (L-INS = 0.30 ± 0.007; H-INS = 0.42 ± 0.006 ng/mL). Interestingly, cows in the L-INS group had higher (P = 0.003) NEFA concentrations compared with cows in the H-INS group (L-INS = 0.57 ± 0.02; H-INS = 0.49 ± 0.02 mmol/L). We observed an insulin-group by parity interaction (P = 0.007) for BHB. The plasma levels of BHB did not differ between primiparous H-INS and L-INS cows (Figure 5.4A). Nevertheless, multiparous L-INS cows tended to have higher ($P \leq 0.10$) BHB concentrations at d 0 and 10 than H-INS cows (Figure 5.4B).

Cows classified into L-INS or H-INS at day 3

Plasma concentrations of Insulin, NEFA, and BHB are illustrated in Figure 5.3B. Cows grouped as L-INS had lower (P < 0.001) plasma insulin when compared with H-INS cows (L-INS = 0.31 ± 0.007; H-INS = 0.42 ± 0.007 ng/mL). Moreover, L-INS cows had higher (P = 0.008) NEFA concentrations compared with H-INS cows (L-INS = 0.56 ± 0.02; H-INS = 0.49 ± 0.02 mmol/L). The plasma concentration of BHB did not differ (P = 0.96) between L-INS and H-INS cows (L-INS = 0.73± 0.02; H-INS = 0.73 ± 0.02 mmol/L).

Cows classified into L-INS or H-INS at day 10

Plasma concentrations of Insulin, NEFA, and BHB are illustrated in Figure 5.3C. Cows classified as L-INS had lower ($P < 0.001$) plasma insulin levels compared with H-INS cows (L-INS = 0.31 ± 0.007; H-INS = 0.41 ± 0.007 ng/mL). Furthermore, cows in the L-INS group had higher ($P = 0.01$) NEFA concentrations compared with cows in the H-INS group (L-INS = 0.61 ± 0.03; H-INS = 0.55 ± 0.03 mmol/L). Differences in BHB plasma levels were not observed ($P = 0.54$) between groups (L-INS = 0.74 ± 0.02; H-INS = 0.73 ± 0.02 mmol/L).

Milk Yield and Composition

To test for the genetic merit of milk production cows grouped as L-INS or H-INS at all sample collection time points (d 0, 3 and 10), PTA for milk yield was evaluated. No significant differences were observed among groups at any time point ($P > 0.51$). Test day milk, FCM, ECM, percent fat, and percent protein at the 1st, 2nd and 3rd DHI tests of cows classified as L-INS or H-INS on d 0, 3 and 10 are presented in Table 5.1 and Supplementary Figure 5.1 and 2.

Cows classified into L-INS or H-INS at day 0

Cows classified as L-INS on d 0 had higher TDM ($P = 0.01$), FCM ($P = 0.005$), and ECM ($P = 0.004$) yields compared with H-INS cows. For TDM, FCM and ECM, we detected an insulin-group by test number interaction ($P < 0.05$), whereby L-INS cows produced higher yields of TDM, FCM, and ECM than H-INS cows on test 1. No differences were observed for percent fat between groups. However, percent protein was higher ($P = 0.02$) for H-INS cows when compared with L-INS cows.

For weekly milk yields, we observed that cows classified as L-INS produced 2.04 kg/d more milk ($P = 0.02$) during the first 12 wk after calving compared with H-INS cows (L-INS = 41.93 ± 0.64, H-INS = 39.89 ± 0.59 kg; Figure 5.5A).

Cows classified into L-INS or H-INS at day 3

Test day milk yield was not affected by insulin-group at d 3. However, FCM tended (P = 0.09) to be higher in L-INS cows when compared with H-INS cows. Furthermore, relative to H-INS cows, ECM was higher (P = 0.007) for L-INS cows. Percent fat was similar among groups. However, percent protein tended to be higher (P = 0.09) for H-INS cows when compared with L-INS cows. Additionally, there was a group by test number interaction (P = 0.05) for percent protein, whereby H-INS cows tended (P = 0.07) to have a higher percent protein than L-INS cows on test 1.

No differences were observed between L-INS and H-INS cows for weekly milk yields (L-INS = 41.26 ± 0.64, H-INS = 40.43 ± 0.62 kg; Figure 5.5B).

Cows classified into L-INS or H-INS at day 10

For TDM and FCM, we observed an insulin-group by parity interaction (P < 0.05). Primiparous L-INS cows had higher TDM and FCM yields compared with primiparous H-INS cows. No differences were observed among multiparous L-INS and H-INS cows. Relative to H-INS cows, ECM was higher (P = 0.005) for L-INS cows. No differences were observed for percent fat between groups. However, percent protein was higher (P = 0.02) for H-INS cows when compared with L-INS cows.

For weekly milk yields, we observed an insulin-group by parity interaction (P = 0.002), in which primiparous L-INS cows produced 4.87 kg/d more milk than primiparous H-INS cows (L-INS = 38.22 ± 1.17, H-INS = 33.35 ± 0.84 kg; Figure 5.6A). No differences were observed among multiparous L-INS and H-INS cows (Figure 5.6B).

Body Condition Score Loss

We evaluated BCS loss in L-INS and H-INS cows on d 0, 3 and 10. L-INS cows lost more (P < 0.001) BCS, regardless of the day, compared with H-INS cows (Figure 5.7A).

Table 5.1. Milk yield and composition from cows classified as low insulin (L-INS) or high insulin (H-INS) based on the median plasma insulin concentration from blood samples collected within 12 h (d 0) from parturition, and on d 3 and 10 after calving at 1st, 2nd, and 3rd DHI test.

	Insulin d 0			Insulin d 3			Insulin d 10		
Item	L-INS LSM±SEM	H-INS LSM±SEM	P^1	L-INS LSM±SEM	H-INS LSM±SEM	P^1	L-INS LSM±SEM	H-INS LSM±SEM	P^1
Milk yield (kg/d)	40.75±0.69	38.41±0.64	0.01	39.98±0.69	38.95±0.67	0.28	40.95±0.74	38.66±0.64	0.02[2]
Primiparous	-	-	-	-	-	-	37.01±1.28	32.26±0.92	0.01
Multiparous	-	-	-	-	-	-	44.91±1.28	45.06±0.92	
FCM (kg/d)	40.59±2.36	37.73±2.31	0.005	40.11±2.36	38.40±2.30	0.09	41.00±2.42	38.65±2.28	0.006[2]
Primiparous	-	-	-	-	-	-	35.95±2.65	30.95±2.48	0.04
Multiparous	-	-	-	-	-	-	46.16±2.41	45.39±2.28	
ECM (kg/d)	44.50±0.70	41.67±0.64	0.004	43.65±0.74	40.88±0.72	0.007	44.09±0.73	40.55±0.68	0.005
Fat (%)	3.81±0.16	3.77±0.16	0.56	3.83±0.16	3.76±0.15	0.39	3.85±0.16	3.77±0.15	0.30
Protein (%)	2.99±0.02	3.07±0.02	0.02	3.01±0.02	3.06±0.02	0.09	2.98±0.02	3.07±0.02	0.003

TDM = Test day milk
FCM = 3.5% Fat-corrected milk
ECM = Energy-corrected milk
[1]*P*-value indicate overall group effect.
[2]*P*-value for the interaction term group × parity was < 0.05.

Figure 5.1. Concentration of insulin in colostrum and plasma of primiparous (n = 20) (A) and multiparous (n = 27) (B) cows at -7, 0 (within 12 h after calving), 3, and 10 d relative to parturition. Different letters indicate a significant difference ($P < 0.05$).

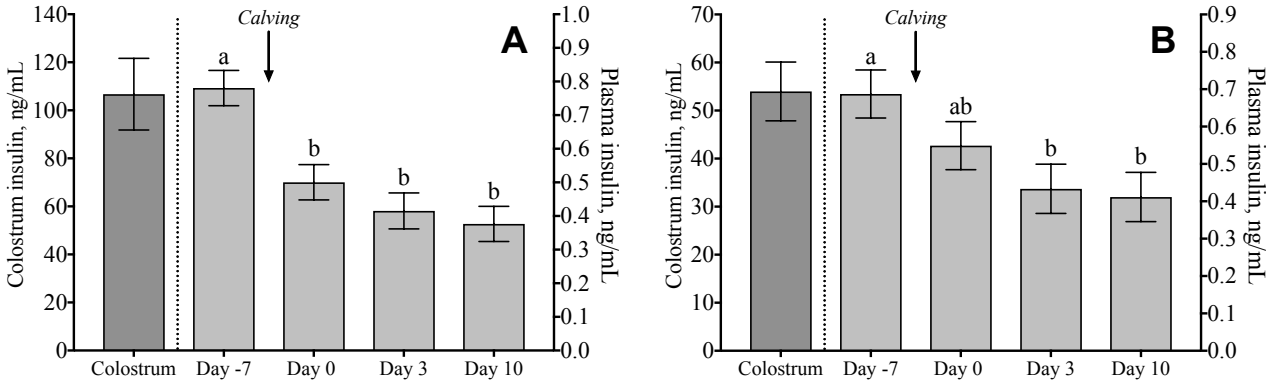


Figure 5.2. Relationship between plasma insulin concentration on day -7 (A) or 0 (B) and colostrum insulin level (n = 47). Spearman's correlation coefficient and *P*-value are illustrated in each graph.

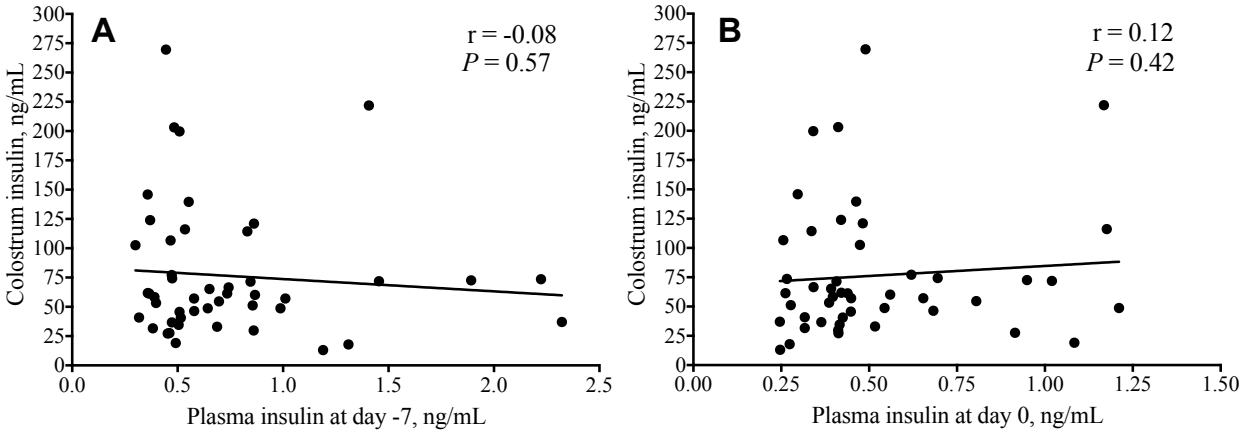


Figure 5.3. Plasma concentration of insulin, fatty acids (NEFA), and β-hydroxybutyrate (BHB) of cows classified as low insulin (L-INS) or high insulin (H-INS) based on the median of plasma insulin measured within 12 h (panel A), at 3 d (panel B) and 10 d (panel C) after calving. *P*-values for the fixed effect of group, time and group × time are included in each graph. Results are reported as LSM ± SEM. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, † $P \leq 0.1$.

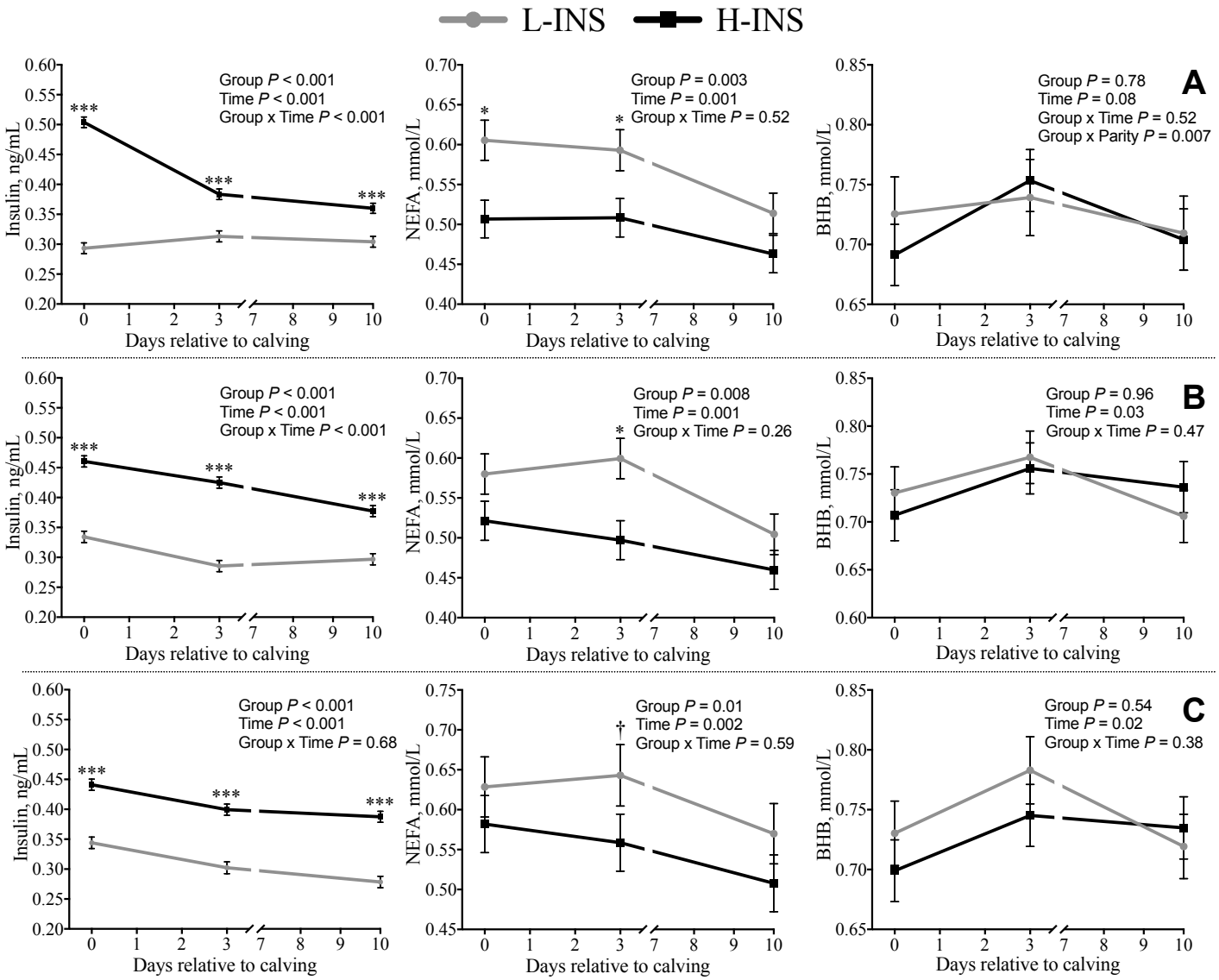


Figure 5.4. Plasma concentration of β-hydroxybutyrate (BHB) of primiparous (A) and multiparous (B) cows classified as low insulin (L-INS) or high insulin (H-INS) based on the median plasma insulin concentration from blood samples collected within 12 h (d 0) after calving. Results are reported as LSM ± SEM. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, † $P \leq 0.1$.

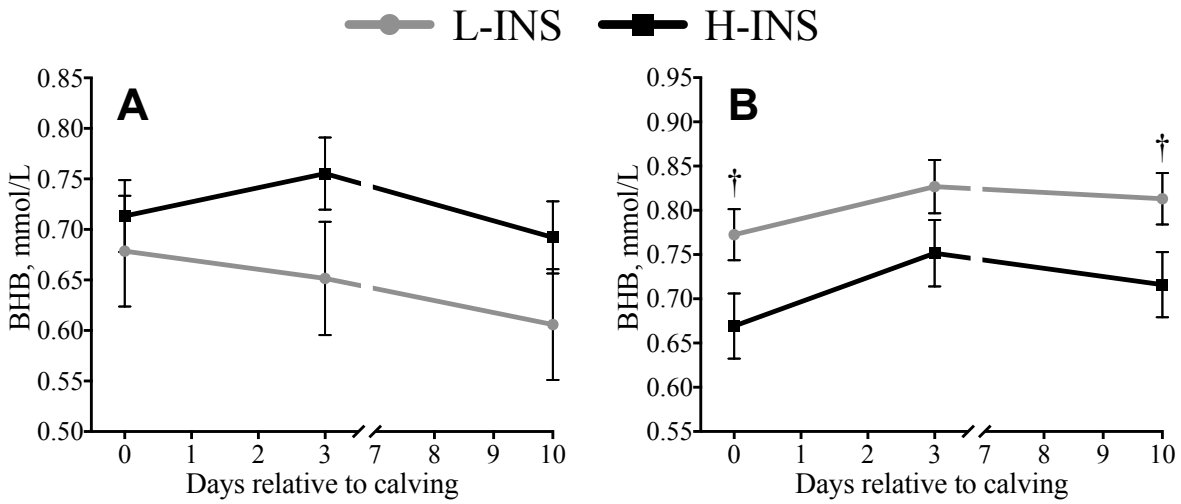


Figure 5.5. Weekly milk yields of cows classified as low insulin (L-INS) or high insulin (H-INS) based on the median plasma insulin from blood samples collected within 12 h (d 0) from parturition (A), on d 3 (B), and d 10 (C) after calving. *P*-values for the fixed effect of group, time, group × time, and group × parity are included in each graph. Results are reported as LSM ± SEM.

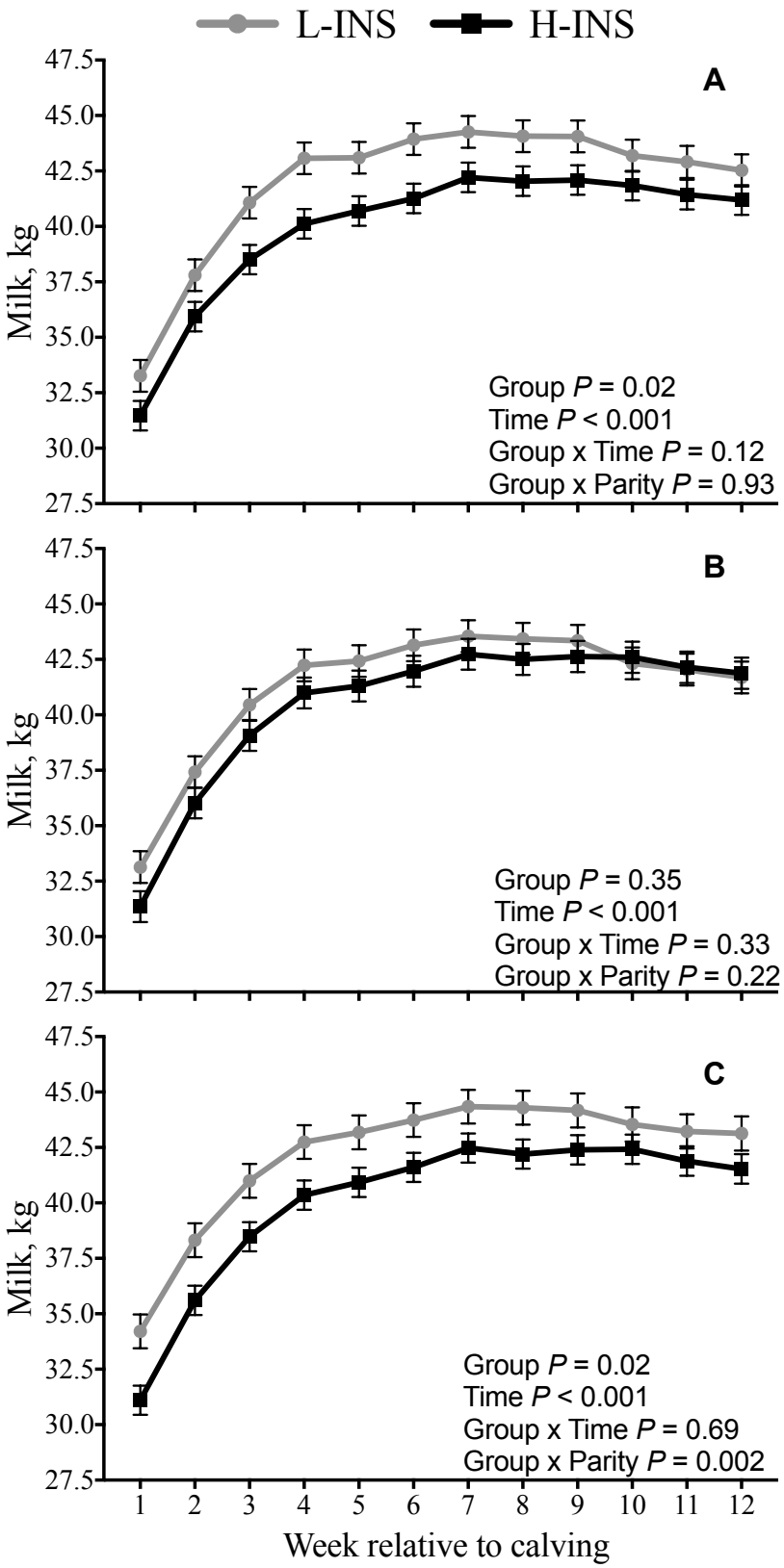


Figure 5.6. Weekly milk yields of primiparous (A) and multiparous (B) cows classified as low insulin (L-INS) or high insulin (H-INS) based on the median of plasma insulin on d 10 after calving. Results are reported as LSM ± SEM.

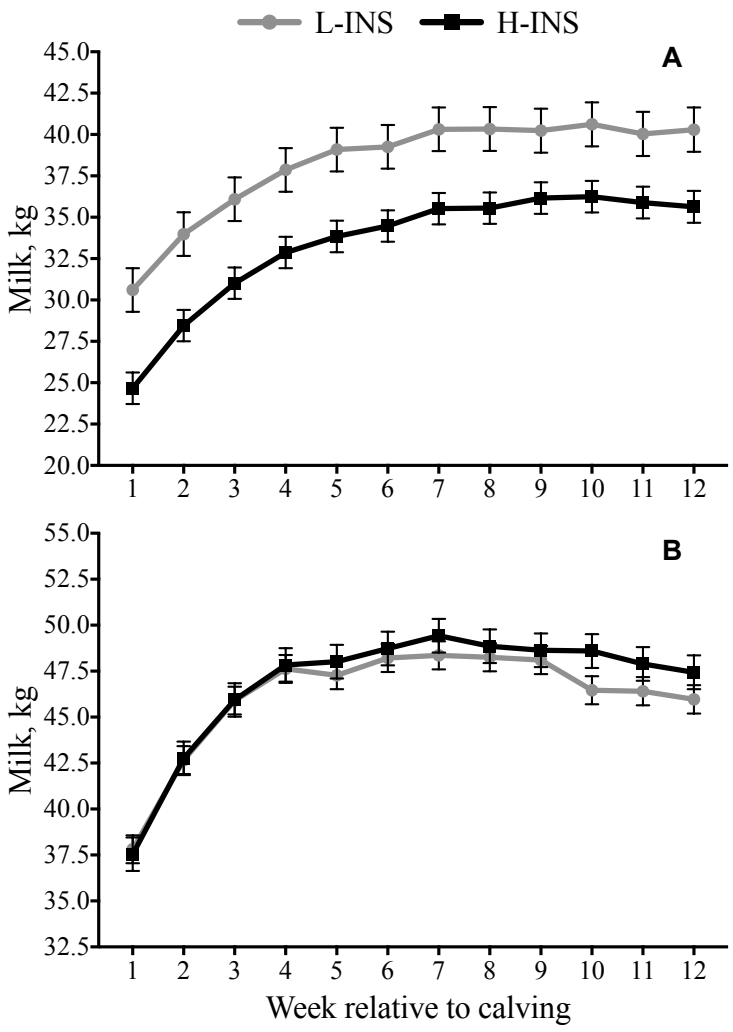
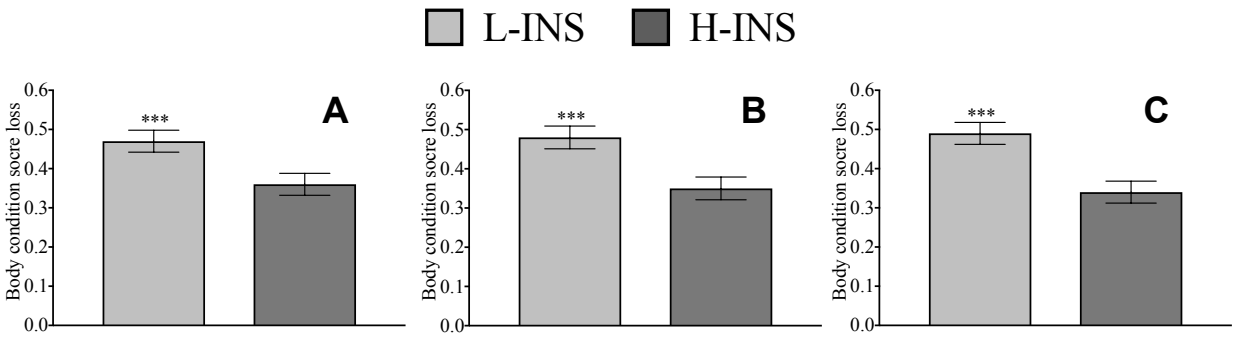


Figure 5.7. Body condition score loss from calving until 35 days in milk of cows classified as low insulin (L-INS) or high insulin (H-INS) based on the median of plasma insulin within 12 h from parturition (A), and on d 3 (B) and d 10 (C) after calving. Results are reported as LSM ± SEM. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, † $P \leq 0.1$.



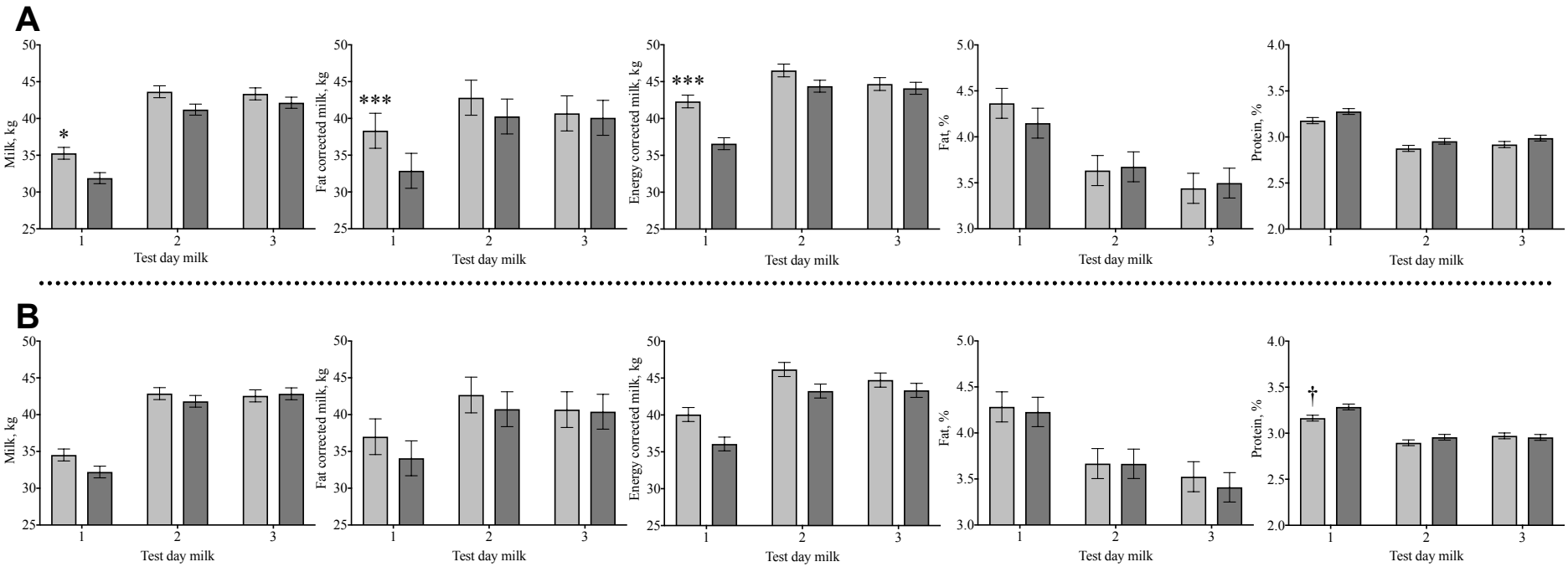
SUPPLEMENTARY INFORMATION

Supplementary Table 5.1. Frequency distribution of each insulin classification group (L-INS or H-INS) for sample collection days 0, 3, and 10.

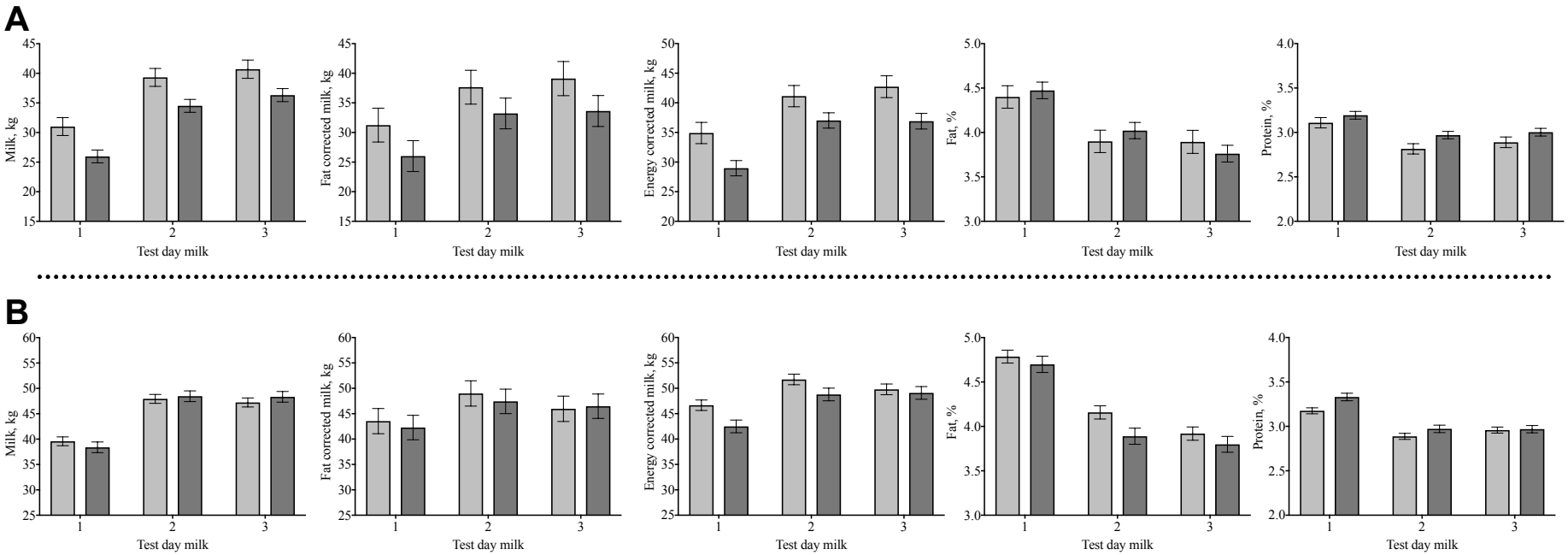
		Group					
		Insulin d 0		Insulin d 3		Insulin d 10	
Group		L-INS	H-INS	L-INS	H-INS	L-INS	H-INS
Insulin d 0	L-INS	133(100%)	0	91(74%)	32(26%)	91(68%)	42(32%)
	H-INS	0	134(100%)	34(27%)	92(73%)	42(32%)	91(68%)
Insulin d 3	L-INS	91(73%)	34(27%)	125(100%)	0	97(78%)	28(22%)
	H-INS	32(26%)	92(74%)	0	124(100%)	21(17%)	103(83%)
Insulin d 10	L-INS	91(68%)	42(32%)	97(82%)	21(18%)	133(100%)	0
	H-INS	42(32%)	91(68%)	28(21%)	103(79%)	0	133(100%)

Cows were grouped according to plasma insulin concentration based on the median as low insulin (L-INS) or high insulin (H-INS) on d 0 (median = 0.35 ng/mL), 3 (median = 0.32 ng/mL), and 10 (median = 0.30 ng/mL).

Supplementary Figure 5.1. Milk yield and composition from cows classified as low insulin (L-INS; light gray) or high insulin (H-INS; dark gray) based on the median plasma insulin concentration from blood samples harvested within 12 h from parturition (panel A), and on d 3 (panel B) after calving at 1st, 2nd and 3rd DHI test. Results are reported as LSM ± SEM. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, † $P \leq 0.1$.



Supplementary figure 5.2. Milk yield and composition from primiparous (panel A) and multiparous (panel B) cows classified as low insulin (L-INS; light gray) or high insulin (H-INS; dark gray) based on the median plasma insulin concentration from blood samples harvested on d 10 at 1st, 2nd and 3rd DHI test. Results are reported as LSM ± SEM.



DISCUSSION

Our main hypothesis was that low plasma insulin during early postpartum would be associated with increased milk yield. In support of our hypothesis, we observed that cows with low plasma insulin (L-INS cows) produced more milk, FCM, and ECM when compared with cows with high plasma insulin levels (H-INS cows).

Due to its anabolic action, insulin stimulates the uptake of glucose, amino acids, and fatty acids into body tissues, while it inhibits metabolic pathways for glucose production (Hart, 1983; De Koster and Opsomer, 2013). It is well known that in postpartum dairy cows, insulin secretion is diminished to prioritize the use of glucose for milk production; this is considered one of the major endocrine adaptations of dairy cows from late pregnancy to early lactation (Hart, 1983; Bell, 1995; Bell and Bauman, 1997). Here, we described the plasma insulin concentrations before calving and during early lactation. In agreement with the literature, we observed that circulating insulin is suppressed after parturition to assure glucose availability to the insulin-independent mammary gland for production of copious amounts of milk.

Previous studies have shown that administration of insulin decreases milk yield in Holstein cows (Kronfeld et al., 1963; Schmidt, 1966; Winkelman and Overton, 2013). Schmidt (1966) observed that primiparous cows that received repeated administrations of a short-acting insulin had reduced milk yield and lactose content of milk. Similarly, Kronfeld et al. (1963) observed a decrease in milk yield in cows given repeated injections of long-acting insulin. In the present study, we observed that cows with low plasma insulin concentrations produced significantly more milk and higher FCM and ECM compared with cows with high plasma insulin. Moreover, our results are in accordance with the study conducted by Lucy et al. (2009). In that study, North American Holstein cows produced more milk when compared with New Zealand Holstein-Friesian cows. They observed that New Zealand Holstein-Friesian cows tended to have higher plasma insulin concentration 1 wk prior to calving and numerically higher on the first wk postpartum than North American Holstein cows (Lucy et al., 2009). Therefore, the aforementioned studies support our findings, that low insulin is associated with higher milk

production. We also observed a group by parity interaction when milk yield was analyzed from cows classified as L-INS or H-INS on d 10, where the increase in milk yield was driven by primiparous cows. An explanation for this observation may be the differences in the control of nutrient partitioning that exist between primiparous and multiparous cows, in which first-parity animals promote nutrient utilization into growth (Wathes et al., 2011). Thus, primiparous cows that still have high circulating insulin on d 10 might redirect the use of glucose toward growth.

Increasing milk protein production has been observed in dairy cows that received an exogenous administration of insulin (Kronfeld et al., 1963; Schmidt, 1966; Winkelman and Overton, 2013). Our results on milk protein content are in accordance with the published studies. We observed that cows classified as H-INS produced significantly more protein in milk compared with L-INS cows. Although the observed reduction in milk protein of L-INS cows might be due in part to a dilution effect, it has also been demonstrated that insulin can promote milk protein synthesis (Griinari et al., 1997; Mackle et al., 1999).

As mentioned above, insulin signaling normally promotes triglyceride synthesis in adipose tissue by increasing lipoprotein lipase activity, thus favoring the uptake of fatty acids into adipocytes from circulating lipoproteins (Hayirli, 2006). Here, the described lipogenic and anti-lipolytic effects of insulin were reflected by plasma NEFA levels. We observed that cows with low plasma insulin had significantly higher concentrations of circulating NEFA. In support, L-INS cows lost significantly more BCS than did H-INS cows. Interestingly, BHB plasma levels did not differ between H-INS and L-INS cows. However, it has been shown that the blood concentrations of NEFA and BHB during early lactation have a weak correlation (Ospina et al., 2013; McCarthy et al., 2015). In the study conducted by McCarthy et al. (2015), the authors suggested that other sources of carbon such as lactate or ketogenic amino acids might contribute to BHB synthesis.

Lastly, we measured the relationship between insulin concentrations in colostrum and in plasma at 7 d before the expected day of calving and within 12 h relative to parturition. Insulin in colostrum is known to play a role in postnatal intestinal development (Shehadeh et al., 2006;

Hammon et al., 2013). Ronge and Blum (1988) observed that the colostrum insulin concentration was ~20 times higher than in prepartum plasma and ~35 times higher than in milk (Ronge and Blum, 1988). In addition, a recent study shown that the colostrum insulin concentration was ~80 times higher than in prepartum plasma (Mann et al., 2016). In the present study, we observed that the colostrum insulin concentration was ~100 times higher than in prepartum plasma. However, the transport mechanism of circulating insulin into the mammary gland is not understood. The dramatic difference in insulin concentration between plasma and colostrum suggests that insulin is actively and not passively transported from blood to the mammary gland. Here, we observed no relationship between plasma insulin and colostrum insulin levels, either at -7 d from the expected day of parturition or within 12 h after parturition. However, the study conducted by Mann et al. (2016) revealed a moderate positive correlation between plasma insulin and colostrum insulin on d 1 to 3 prepartum (Mann et al., 2016). Further investigation is needed to elucidate the exact mechanism of insulin uptake by the mammary gland.

There are limitations in the present study that need to be pointed out. One limitation is that the study was limited to a single dairy farm, which restricts the external generalization of the results. Because plasma insulin concentration significantly varies during the day (Lefcourt et al., 1999), it is possible that median of plasma insulin observed in the current study and used to group cows as L-INS or H-INS might have been different if samples were collected at a different time of the day. However, according to the results presented by Lefcourt et al. (1999), plasma insulin levels between 0630 to 1100 h (range of sample collection time of our study) seems to be the most stable period of time during the day for plasma insulin concentration. In addition, on that study, cows were fed a total mixed ration at 0900 h, which is similar feeding time for the cows in the present study (~0800 h). Nevertheless, since our study was conducted in a commercial dairy farm, the exact time of feeding could have varied during the study and thus altering the concentration of insulin in plasma. Therefore, the results presented here need to be interpreted in the light of these limitations.

CONCLUSIONS

In summary, we observed that cows with low plasma insulin during the early postpartum produced more milk, had higher FCM and ECM compared with cows with high plasma insulin. Cows with high insulin concentrations had a higher percentage of milk protein compared with cows with low insulin levels. The results presented here highlight the importance of the depression of postpartum insulin secretion as a key endocrine adaptation to support high milk production.

ACKNOWLEDGEMENTS

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CHAPTER 6: Effects of pegbovigrastim administration on periparturient diseases, milk production, and reproductive performance of Holstein cows*

*Zinicola, M., Korzec, H., Teixeira, A.G.V., Ganda E.K., Bringhenti, L, Tomazi, A. C. C. H., Gilbert, R. O., Bicalho, R.C. Effetcs of pegbovigrastim administration on periparturient dieases, milk production, and reproductive perforamce. J Dairy Sci. In press. https://doi.org/10.3168/jds.2018-14869.

ABSTRACT

The aim of this study was to evaluate the effects of treating Holstein cows with pegbovigrastim on periparturient diseases, milk production, and reproductive performance while exploring the mode of action of an immunomodulatory protein. Cows were randomly allocated into one of two treatments, untreated control (**CTR,** n = 423) and pegbovigrastim (**PEG**, n = 417). At 7 d from the anticipated calving date (d -7), cows allocated to PEG received a subcutaneous injection of 15 mg of pegylated recombinant bovine G-CSF (pegbovigrastim injection, Imrestor, Elanco Animal Health). A second injection was administered within 24 h after calving (d 0). Blood samples were obtained from a subset of cows (CTR, n = 103; PEG, n = 102) at -7 d, and at 0, 3, 7, and 14 d relative to parturition. Samples were used for hemogram and quantification of haptoglobin, non-esterified fatty acids, β-hydroxybutyrate (**BHB**), and trace and macro minerals. Vaginal cytobrush was performed on the same subset cows at d 0, 7 and 14 to assess the relative neutrophil count. Additionally, colostrum samples were collected to measure IgG, IgM, IgA, and lactoferrin concentrations. Postpartum disease occurrence was recorded from calving until 30 DIM. Weekly milk yield was recorded for the first 12 wk after calving. Cows treated with PEG had a 3- to 4-fold increase in circulating polymorphonuclear leukocyte (**PMN**), lymphocyte, and monocyte numbers, with a peak at 3 d after treatment followed by a gradual decline, but the counts remained significantly greater compared with CTR at 14 DIM. The administration of PEG did not affect the incidence of clinical and subclinical mastitis, retained fetal membranes, metritis, puerperal metritis, and endometritis. Primiparous cows treated with PEG tended to have lower odds of developing hyperketonemia than CTR (OR = 0.57, 95% CI: 0.23 to 1.42). Cows treated with PEG had higher odds of being diagnosed with lameness within 30 DIM compared with CTR (OR = 1.79, 95% CI: 1.16 to 2.76). However, there were no significant differences by 60 DIM. Treatment with PEG increased the odds of displaced abomasum (OR = 8.27, 95% CI: 1.02 to 66.6). Cows treated with PEG had higher odds of being diagnosed with 1 or more clinical diseases compared to CTR cows (OR = 1.39, 95% CI: 1.02 to 1.90). We observed no differences on linear scores or milk composition between treatments. Furthermore, primiparous cows treated

with PEG produced more milk than CTR primiparous cows during the first 12 wk postpartum (PEG = 37.51 ± 0.66; CTR = 35.91 ± 0.65 kg), but no differences were observed on ECM. Treatment did not alter reproductive performance. Additionally, cows diagnosed with metritis and/or puerperal metritis and treated with PEG tended to have higher proportion of neutrophils in the vaginal mucosa when compared with CTR-metritic cows. Although PEG treatment increased circulating PMN, monocyte, and lymphocyte numbers as expected, it was detrimental to cow health because it increased morbidity.

Keywords: Holstein, mastitis, metritis, pegbovigrastim

INTRODUCTION

During the early postpartum period, dairy cows can experience several infectious and metabolic disorders (Seegers et al., 2003; Duffield et al., 2009; Dubuc et al., 2010). These conditions are costly to the producer and compromise the welfare of the animals (Fetrow et al., 2000; Dubuc et al., 2011). Despite decades of research dedicated to advancing knowledge and aiding in the prevention of periparturient disorders (e. g. mastitis, retained placenta, and metritis) their incidence still considerably high (Ribeiro et al., 2013). Thus, novel treatment options and control programs are required for dairy cattle.

Mastitis is highly prevalent in dairy cows and arguably the most important disease for the dairy industry worldwide (Seegers et al., 2003; Grohn et al., 2004; Cha et al., 2011). Treatment and prevention of mastitis have been reported to be the principal reason for antibiotic usage in dairy farms, accounting for 80% of the antimicrobial dugs used in the dairy industry (Pol and Ruegg, 2007). Intensive use of antimicrobials provides opportunity for antimicrobial resistance to develop in mastitis pathogens (Oliver and Murinda, 2012) and increases the risk of residue violation (Ruegg and Tabone, 2000). Therefore, efforts have been made to manage mastitis and prevent the indiscriminate use of antibiotics in dairy farms (Schreiner and Ruegg, 2003; Landin et al., 2015; Ganda et al., 2016).

Neutrophils are the main cell line of defense involved in bacterial clearance after uterine (Hussain, 1989) and mammary gland (Paape et al., 2002) infection, and play a major role in placental release (Kimura et al., 2002). However, impairment of neutrophil function during the peripartum period has been well documented and is a key contributing factor to the high incidence of postpartum infectious diseases (Kehrli et al., 1989; Kimura et al., 1999; Rinaldi et al., 2008). Indeed, postpartum uterine diseases and mastitis have been associated with decreased neutrophil function (Waller, 2000; Kimura et al., 2002; Hammon et al., 2006). Additionally, it has been shown that greater influx of neutrophils into the uterus during the early postpartum period is associated with improved uterine health and reproductive performance (Gilbert and Santos, 2016).

Granulocyte colony stimulating factor (**G-CSF**) is an endogenous hematopoietic growth factor that stimulates the production and differentiation of neutrophils by progenitor cells in the bone morrow (Nagata, 1989). In humans, the prophylactic use of G-CSF has been demonstrated to be effective in reducing the incidence of febrile neutropenia — a frequent event observed in cancer patients treated with chemotherapy and characterized by neutrophil numbers below normal values (Cooper et al., 2011).

Recently, a commercially available recombinant bovine G-CSF (pegbovigrastim, Imrestor, Elanco Animal Health) was shown to increase the concentrations of circulating neutrophils and enhanced in vitro myeloperoxidase (**MOP**) release from neutrophils of periparturient cows (McDougall et al., 2017). However, neutrophil phagocytic activity, oxidative burst, and MOP function did not differ between treated and untreated cows (McDougall et al., 2017). Similar results were observed in a previous study (Kimura et al., 2014).

Recent studies have evaluated the effect of pegbovigrastim (**PEG**) administration with the objective of reducing the occurrence of clinical mastitis (**CM**) in postpartum Holstein cows (Hassfurther et al., 2015; Canning et al., 2017; Ruiz et al., 2017). In the study conducted by Hassfurther et al. (2015), cows treated with PEG at 10 and 20 μg/kg experienced significantly

fewer cases of CM (9/54 and 5/53, respectively) compared with control cows (18/53). Canning et al. (2017) evaluated the effect of PEG on postpartum CM incidence from 4 dairy farms located in different states of the United States. They observed that treated cows had an overall (across all dairies) 35% reduction of CM incidence compared with controls. Moreover, a reduction in CM incidence of 25% was reported in cows treated with PEG relative to controls (Ruiz et al., 2017).

We hypothesized that administration of PEG would increase neutrophil numbers in blood. Also, we expected that treated cows would have a reduced incidence of postpartum disorders. Thus, the objectives of the present study were to evaluate the effect of PEG administration on (1) circulating blood cells; (2) the incidence of mastitis (clinical and subclinical) and uterine diseases; and (3) production and reproductive performance. Moreover, serum metabolic markers [non-esterified fatty acids (**NEFA**) and BHB], plasma minerals, systemic inflammation (haptoglobin), and the concentrations of immunoglobulins and lactoferrin in colostrum were assessed.

MATERIALS AND METHODS

Ethics Statement

The research protocol was reviewed and approved by the Cornell University Institutional Animal Care and Use Committee (protocol number 2016-0028). The methods were carried out in accordance with the approved guidelines.

Farm and Experimental Design

The study was conducted in a large commercial dairy farm located near Ithaca, New York. The farm milked ~3,800 Holstein cows thrice daily in a rotary parlor with integrated milk meters that record individual production at every milking (DeLaval, Tumba, Sweden). The cows were housed in free stall barns, with concrete stalls bedded with manure solids treated with quicklime (CaO). The diet was formulated to meet or exceed the NRC nutrient requirements for lactating Holstein cows weighing 650 kg and producing 45 kg of 3.5% FCM (NRC, 2001). When

a cow demonstrated signs of calving, it was moved to an individual maternity pen for delivery, where trained farm personnel assisted with parturition as needed, and then transferred to a postpartum pen after calving. Calves were removed from their dams immediately after birth. The farm reproductive management used for the first service a Presynch-Ovsynch protocol in combination with estrus detection and artificial insemination (Pursley et al., 1995; Moreira et al., 2001). After the first service, cows were submitted to the Resynch (Fricke et al., 2003) protocol, and were allowed to be inseminated at detected estrus. A voluntary waiting period of 50 days was used. Estrus was detected based only on electronic activity sensors (Alpro, DeLaval, MO) worn around the neck. Pregnancy was diagnosed on d 39 ± 3 after AI via rectal examination. Cows diagnosed as pregnant were reexamined 21 d later for detection of viable pregnancy on d 60 ± 3. Pregnant cows were considered as diagnosed pregnant at the first rectal examination. Pregnancy loss was defined as the absence of a viable pregnancy at the second rectal examination.

A randomized clinical trial design was used. In total, 885 primiparous and multiparous cows were enrolled in the study from September 2016 to December 2016. At enrollment, all cows were scored for body condition (**BCS**) (Edmonson et al., 1989) and rectal temperature (**RT**) was recorded. To be enrolled in the study, cows had to meet the following inclusion criteria: BCS > 2.75 and < 4, non-lame, and RT < 40.0 °C. Cows with a gestation length > 265 d were eligible to be enrolling in the study. Treatment allocation was done by parity in two separate randomization sheets on the d of the first pegbovigrastim injection. Multiparous cows were blocked by previous 305-d mature equivalent lactation yields (**305-ME**). Cows were then randomly allocated to one of two treatment groups: untreated control (**CTR,** n = 423) and pegbovigrastim (**PEG**, n = 417). At 7 d from the anticipated calving date, cows allocated to PEG received a subcutaneous injection containing 15 mg of pegylated recombinant bovine G-CSF in 2.7 mL pre-filled syringes (pegbovigrastim injection, Imrestor, Elanco Animal Health). A second injection was administered within 24 h after calving. Subjects that did not receive the first (n = 10) or the second (n = 35) dose of PEG were excluded from the study. Therefore, a total of 840

cows (primiparous, n = 238; multiparous, n = 602) were used. All treatments were performed by the researchers. To avoid biases, treatment identification was not listed in the data collection forms or in the on-farm herd management software Dairy Comp 305 (**DC305**; Valley Agricultural Software, Tulare, CA).

Animal Sampling

Blood samples were drawn into 10-mL vacutainer K_2 EDTA blood collection tubes (BD Vacutainer, Franklin Lakes, NJ) from a subset of cows (PEG, n = 102; 37 primiparous, 65 multiparous; CTR, n = 103; 39 primiparous, 64 multiparous) from coccygeal vessels at 7 d prior to the expected calving date (d -7; right before the first treatment injection), within 24 h after parturition (d 0; right before the second treatment injection), 3 DIM, 7 ± 3 DIM (d 7), and 14 ± 3 DIM (d 14). After collection, samples were immediately placed on ice. Samples were used for hemogram determination within 6 h of blood collection using a Vet hemogram instrument (Heska – Hemature^{tm}, Loveland, CO), and plasma was obtained by centrifugation at 2,000 × *g* for 15 min at 4 °C, and frozen at -80 °C. Using the same subset of the study population, the relative neutrophil count of the reproductive tract was assessed in the vaginal mucosa from samples collected at d 0, 7, and 35 ± 3 DIM. Briefly, restrained cows had their perineum area cleaned and disinfected with 70% ethanol, and samples were collected by introducing a cytobrush into the vagina. Slides for cytological examination were prepared by rolling the cytobrush onto a clean-labeled glass microscope slide, air-dried immediately on farm and stained at the laboratory. Each slide was exanimated at 400× magnification by a single observer. The observer counted a total of 200 cells from each slide at 2-4 different locations and the percentage of neutrophils among total cells was calculated. For each cow, RT and BCS were measured at the blood collection time points. BCS was also scored at d 35. Additionally, metritis and endometritis diagnosis was performed using a Metricheck device (Metricheck, SimcroTech, Hamilton, New Zealand) in all study cows on d 7 and 35, respectively.

Detection of subclinical mastitis (**SCM**) was performed by the researchers at 10 ± 3 DIM

for all cows enrolled in the study, by using a California Mastitis Test (**CMT**) after milking and the results were recorded for each quarter of the udder. Furthermore, composited milk samples were collected from a subset of cows (primiparous: PEG, n = 23; CTR, n = 22; multiparous: PEG, n = 87; CTR, n = 97) at 7 ± 3, 14 ± 3, 21 ± 3 and 28 ± 3 DIM by the official Ithaca DHI for cow-level SCC analysis. In addition, DHI performed monthly visits to the farm and collected milk samples from all cows enrolled in the study to evaluate the monthly incidence of SCM. Linear somatic cells score was calculated as follows:

$$SCS = [\ln (SCC / 100 \times 10^3) / 0.693147] + 3.$$

Lastly, a convenience sampling method was used to collect colostrum samples from 200 cows (100/treatment) to quantify immunoglobulin (**Ig)** G, A, and M, and lactoferrin concentrations. Briefly, teat ends were cleansed and 10 mL of colostrum from each quarter was sterilely collected into 50-mL falcon tubes (VWR international, Radnor, PA), placed on ice and transported to the laboratory and frozen at -20 °C. Colostrum samples were collected before blood collection on d 0.

Plasma and Colostrum Analysis

Plasma concentrations of NEFA (NEFA-C® kit; Wako Pure Chemical Industries, Richmond, VA) and BHB [(Williamson and Mellanby, 1974), kit#310-UV, Sigma-Aldrich, St. Louis, MO] were determined by colorimetric methods. Haptoglobin concentrations were determined using a colorimetric procedure as previously described (Bicalho et al., 2014). The intra-assay and inter-assay coefficients of variation for the assays were <8.5% and <10.5%, respectively. Samples collected at d 0 from cows entering their third or greater lactation (PEG, n = 33; CTR, n = 30) were sent to the Veterinary Diagnostic Laboratory at Iowa State University (Ames, IA) for analysis of Ca, Cu, Fe, K, Mg, Mn, P, Se, and Zn. The concentrations of plasma minerals were analyzed by using an inductively coupled plasma mass spectrometry system (Varian/Bruker 820 ICP-MS, Bruker Corporation, Fremont, CA) through separation of analyte ions from spectral interferences.

Enzyme-linked immunosorbent assays were used to quantify colostrum IgG (Immuno-Tek Bovine IgG ELISA Kit, ZeptoMetrix Corporation, Buffalo, NY), IgM, IgA, and lactoferrin (Bethyl Laboratories, Bovine ELISA Kit) concentrations. Colostrum samples were thawed and homogenized, and aliquots of 1.5 mL were centrifuged at 10,000 × *g* for 15 min at 4 °C, then the supernatant was collected for analysis. A serial dilution steps (100 × 100 × 100) were performed to achieve a final total dilution of 1:1,000,000. The intra-assay and inter-assay coefficients of variation for the ELISAs were <7% and <9.5%, respectively.

Disease Definitions

Stillbirth was defined as a dead calf at birth. Dystocia was defined as a calving that required assistance from farm personnel. Retained placenta (**RP**) was defined as cows that failed to release the fetal membranes within 24 h after calving (Kelton et al., 1998). Metritis diagnosis was performed by the researchers at 7 ± 3 d using the Metricheck device and defined as the presence of fetid, watery, red-brown uterine discharge. Puerperal metritis diagnosis was performed by the researcher and by trained farm personnel. Researcher diagnosed puerperal metritis was defined as cows having metritis with RT > 39.5°C (Sheldon et al., 2006). Farm diagnosed puerperal metritis was performed daily by trained farm employees. Cows were flagged for a physical examination when showing signs of dullness and depression or when a milk deviation of more than 4.5 kg was detected. At the physical examination cows were considered as puerperal metritis when a fetid, watery and red-brown uterine discharge combined with systemic illness was observed. For the purpose of this study, cows diagnosed with puerperal metritis by the research team and/or by farm employees were grouped together and considered positive for puerperal metritis. Furthermore, information regarding puerperal metritis diagnosis was not exchanged between the researchers and farm personnel. For some analysis, metritis and puerperal metritis were combined, and named metritis-PM. Fever was considered as cows having RT > 39.5°C on d 7 or 14 postpartum. Endometritis diagnosis was performed at 35 ± 3 DIM using the Metricheck device; the vaginal discharge was scored using a 0 to 3 scale (0 = no secretion

material retrieved and/or clear mucus, 1 = flecks of purulent material within otherwise clear mucus; 2 = <50% of purulent material in the vaginal discharge, 3 = >50% of purulent material with/without fetid discharge); cows with a score of ≥2 were considered as diagnosed with clinical endometritis. Displaced abomasum (**DA**) diagnosis was made by farm personnel and confirmed by veterinarians. Lameness was defined as cows with clinical manifestation of abnormal locomotion detected by farm personnel. Hyperketonemia (**HYK**) was defined as a plasma BHB concentration ≥1.2 mmol/L on d 3, 7 or 14 (McArt et al., 2012). Additionally, subclinical hypocalcemia (**SCH**) was defined as a plasma Ca concentration < 7.5 mg/dL (Goff et al., 1996; Neves et al., 2018).

Clinical mastitis was evaluated during the first 30 DIM and defined as the presence of abnormal milk, such as watery appearance, flakes and clots in milk during forestripping detected at each milking by trained farm employees. Once diagnosed in the milking parlor, the cow was immediately sorted on the DeLaval milking overview screen, which triggered her to be sorted through the DeLaval sort gate. Once the cow was sorted, a trained farm employee obtained a sample from the infected quarter(s). All mastitic cows were cultured using an on-farm culture system (Accumast, FERA Animal Health LCC, Ithaca, NY). On a daily basis, CM cases were scored by the research team using the traditional severity score system: mild (abnormal milk with no quarter swelling), moderate (abnormal milk with a swollen quarter), and severe (abnormal milk with a swollen quarter and systemic illness). Subclinical mastitis was evaluated by CMT at 10 ± 3 DIM. The CMT reaction of each quarter was recorded with a score ranging from 0 to 3, with 0 indicating no reaction, 1 being a trace (approx. 200,000 to 500,000 SCC), and 2 or 3 as a CMT positive; SCM was defined as cows detected with a CMT score ≥1. In addition, cows with SCC >200,000 from milk samples collected by DHI were defined as having SCM. A “hospital pen trip” was defined as cows moved by farm personnel to the hospital pen for therapy with antimicrobials, anti-inflammatories, supportive therapy, or any therapy that required the milk and/or the meat to be withheld.

Statistical Analysis

To evaluate the effect of treatment on the proportion of cows affected by clinical diseases, the following diseases were combined for analysis: RP, metritis, puerperal metritis, endometritis, DA, lameness, and CM.

Descriptive statistics were performed in JMP Pro 11 (SAS Institute Inc., NC), using the ANOVA and chi-square functions for continuous and categorical data, respectively. For analyses of repeated measurements, such as white blood cells (**WBC**), PMN, monocytes (**MONO**), lymphocytes (**LYM**), % neutrophils present in the reproductive tract, plasma metabolites, LS, and milk yield and composition, we used general mixed linear models with the MIXED procedure of SAS (version 9.4; SAS/STAT, SAS Institute Inc., Cary, NC). The independent variables offered to the models were: treatment, time, parity (primiparous vs multiparous), BCS at enrollment, dystocia, stillbirth, and RT at enrollment. To evaluate the effect of treatment on blood cells, vaginal cytology, and milk production among cows diagnosed with the following events: metritis, puerperal metritis, metritis-PM, mastitis, clinical diseases, and cows diagnosed pregnant or open at 120 DIM, the following variables were offered to the models: treatment, event, time, parity, dystocia, stillbirth, BCS at enrollment, and RT at enrollment. Two-way and three-way interaction terms between independent variables were offered to the models. For the hemogram data, baseline values of blood cell parameters were treated as covariate variables. Normality and homoscedasticity of residuals were assessed using residual plots. Several covariance structures were tested (unstructured, autoregressive 1, compound symmetry), and the one with the minimum Akaike information criterion was chosen. For all models, Tukey's honest significance test for multiple comparisons was used. Data are reported as LSM ± SEM unless otherwise stated.

The chi-square test was used to compare the incidence of the evaluated periparturient diseases. For the chi-square test, we followed the assumption that no cell should have an expected frequency of less than 5; when the assumption was not satisfied, Fisher's exact test was used. The effect of treatment on the odds ratio (**OR**) of binary response variables was analyzed

by logistic regression using the GLIMMIX procedure of SAS and the fixed effects of treatment, parity, BCS at enrollment, RT at enrollment, and the interaction term treatment by parity were included as independent variables in the statistical models. A Poisson regression model was used to evaluate the effect of treatment on the number of clinical mastitis cases using the PROC GENMOD procedure in SAS. To assure validity of the Poisson model, goodness of fit was evaluated with the Pearson Chi-square test. Treatment, parity and the interaction term treatment by parity were included as fixed effects. Controls were considered as the reference group.

The effect of treatment on the hazard of pregnancy and herd removal during the first 180 DIM, and days to the first service during the first 120 DIM were analyzed by Cox's Proportional Hazard using the PHREG procedure in SAS. Variables offered to the models included treatment, parity, BCS at enrollment, and interactions. To illustrate the effect of treatment on reproductive performance, herd removal, and days to first service, Kaplan–Meier survival analysis was carried out using MedCalc version 11.5.1.0 software (MedCalc Software). Body condition score loss was defined as BCS at calving minus BCS at d 35, and assessed using the GLIMMIX procedure of SAS. Colostrum concentrations of IgG, IgA, IgM, and lactoferrin, and plasma minerals were analyzed with the GLIMMIX procedure of SAS. Lastly, multivariable analysis was conducted to assess the effect of treatment on pregnancy per AI (**P/AI**) at first service and pregnancy loss after first AI using the GLIMMIX procedure of SAS. Variables offered to the model included treatment, parity, BCS at enrollment, AI protocol, sire, inseminator, and the interaction term treatment by parity.

For all models, variables and their respective interaction terms were retained in the model when $P \leq 0.15$. Significances were considered when $P \leq 0.05$ or a trend if $0.05 < P \leq 0.10$.

RESULTS

Descriptive Data

Descriptive data per treatment group regarding the number of cows enrolled in the study, average parity, number of primiparous and multiparous, BCS at enrollment, RT at enrollment,

length of the dry period in days for multiparous cows, 305-ME, sire PTA for primiparous cows, days of gestation, and the incidences of twins, male calves, and stillbirth are presented in Table 6.1. No differences were detected in any of these variables (Table 6.1).

Days from First to Second Administration of Pegbovigrastim

Figure 6.1 illustrates the interval between the first (7 d prior to the expected day of parturition) and second dose (within 24 h after calving) of PEG. Ninety percent of the cows received the 2 doses of PEG within 14 d of one another (Figure 6.1A). On average, cows received both doses of PEG with an interval of 8.83 d, with a SD of 4.62 d and a range from 0 to 26 d (Figure 6.1A). The frequency distribution of the interval between the first and second dose of PEG was also evaluated by parity. Ninety percent of primiparous cows received the 2 doses of PEG within 14 days of one another (Figure 6.1B). On average, primiparous cows received the 2 doses of PEG with an interval of 8.62 d, with a SD of 4.29 d and a range from 0 to 19 d (Figure 6.1B). Ninety-one percent of multiparous cows received the 2 doses of PEG within 14 d of one another (Figure 6.1C). On average, multiparous cows received the 2 doses of PEG with an interval of 8.92 d, with a SD of 4.75 d and a range from 0 to 26 d (Figure 6.1C).

Effect of Pegbovigrastim on Hemogram Parameters

The effects of PEG on the concentration of WBC, LYM, PMN, and MONO are presented (Figure 6.2A-D). The Vet hemogram instrument used recorded numbers of PMN and did not differentiate between neutrophils, basophils and eosinophils, although neutrophils represent over 90% of the PMN (George et al., 2010). Treatment affected ($P \leq 0.001$) the concentrations of WBC, LYM, PMN and MONO. Compared to CTR, treated cows had greater ($P \leq 0.001$) blood counts of WBC (PEG, 21.12 ± 0.42; CTR, 9.90 ± 0.41 × 10^3/μL), LYM (PEG, 7.87 ± 0.16; CTR, 4.10 ± 0.16 × 10^3/μL), PMN (PEG, 10.41 ± 0.25; CTR, 4.86 ± 0.25 × 10^3/μL) and MONO (PEG, 2.81 ± 0.07; CTR, 0.95 ± 0.07 × 10^3/μL) at 0, 3, 7 and 14 d relative to the first PEG administration (Figure 6.2A-D).

Concentrations of WBC, LYM, PMN and MONO were evaluated for cows diagnosed with and without metritis-PM and mastitis, and for open and pregnant cows at 120 DIM among and within treatment groups (Figure 6.3A-C and Supplementary Table 6.1). Overall, PEG cows had greater ($P \leq 0.001$) WBC, LYM, and MONO when compared to CTR cows, irrespective of the health condition (metritis-PM/mastitis/no metritis-PM or mastitis) or reproductive status (pregnant/open). Cows treated with PEG and diagnosed with metritis-PM had smaller ($P \leq 0.05$) circulating WBC, PMN, LYM and MONO on d 3 compared to PEG cows without metritis-PM (Figure 6.3A). Furthermore, PEG cows diagnosed with metritis-PM had smaller ($P \leq 0.05$) WBC and PMN on d 7 compared with PEG non-metritic cows (Figure 6.3A). Additionally, WBC concentration on d 14 tended to be smaller ($P \leq 0.10$) in PEG-metritic cows when compared to non-metritic PEG-treated cows (Figure 6.3A). Relative to cows diagnosed with mastitis and treated with PEG, PEG cows without mastitis tended to have smaller ($P \leq 0.10$) WBC and had fewer ($P \leq 0.05$) LYM and MONO concentrations on d 0 (Figure 6.3B). In addition, PEG cows diagnosed with mastitis had smaller ($P \leq 0.05$) WBC, PMN, LYM and MONO on d 3 when compared to PEG non-mastitic cows (Figure 6.3B). Lastly, pregnant cows from the PEG group had greater ($P \leq 0.05$) LYM and MONO concentrations on d 3 compared to PEG open cows (Figure 6.3C). Statistical differences were not observed for hemogram parameters within CTR cows for any health condition (metritis-PM/mastitis/ no metritis-PM or mastitis) or for reproductive status (pregnant/open) (Figure 6.3A-C).

Counts of WBC, LYM, PMN and MONO were also evaluated for cows diagnosed with and without clinical diseases. Cows treated with PEG had greater ($P \leq 0.002$) WBC and PMN concentrations when compared to CTR cows, regardless of the health condition (clinical diseases/ no clinical disease); more detailed information can be found in Supplementary Figure 6.1 and Supplementary Table 6.1.

Effect of Pegbovigrastim on Plasma Metabolites and Minerals

Treatment with PEG did not affect the plasma concentrations of NEFA, BHB, and

haptoglobin (Figure 6.4). No differences were observed in plasma minerals between the PEG and CTR groups (Supplementary Table 6.2).

Effect of Pegbovigrastim on Vaginal Neutrophil Counts

Treatment with PEG did not affect the relative counts of neutrophils present in the vaginal mucosa (Figure 6.5A). We also evaluated the effect of treatment on the relative counts of neutrophils present in the vaginal mucosa among cows diagnosed with metritis-PM (Figure 6.5B). We detected a treatment by metritis-PM interaction ($P = 0.003$), where PEG cows diagnosed with metritis-PM tended to have more relative neutrophil counts compared with CTR cows with metritis-PM (PEG-metritis-PM, 55.75 ± 5.01; CTR-metritis-PM, 43.38 ± 4.98 %; $P = 0.08$). Additionally, PEG cows diagnosed with metritis-PM had more ($P \leq 0.05$) relative counts of neutrophils on d 7 than CTR cows with metritis-PM for the same day (Figure 6.5B). Cows diagnosed with metritis-PM tended ($P \leq 0.10$) and had greater ($P \leq 0.05$) relative counts of neutrophils at d 0 and 7 compared to cows without metritis-PM, respectively (Supplementary Figure 6.2A). Additionally, cows diagnosed with endometritis had greater ($P \leq 0.05$) relative counts of neutrophils at d 35 compared to cows without endometritis (Supplementary Figure 6.2B). We also evaluated the relative counts of neutrophil present in the vaginal mucosa of pregnant and non-pregnant cows at 120 DIM at d 0, 7 and 35. Pregnant cows had greater percentage of neutrophils at d 0 ($P < 0.01$) and 7 ($P < 0.001$) than non-pregnant cows (Supplementary Figure 6.2C).

Effect of Pegbovigrastim on Periparturient Diseases and Removal from the Herd

The effects of PEG on the evaluated periparturient diseases are presented in Table 6.2. Treatment did not affect the incidence of stillbirth, dystocia, RP, metritis, puerperal metritis, metritis-PM, endometritis, CM, SCM at d 7, 10, 14, 21, and 28, and at first and second DHI test. No differences were detected on postpartum fever between the PEG and CTR groups. A treatment by parity interaction ($P = 0.05$) was observed for HYK incidence. Relative to CTR,

primiparous cows treated with PEG tended ($P = 0.08$) to have lower odds of HYK (OR = 0.57, 95% CI: 0.23 to 1.42). Additionally, PEG cows had higher odds ($P = 0.05$) of being diagnosed with DA compared to CTR cows (OR = 8.27, 95% CI: 1.02 to 66.66). The odds of lameness were higher ($P = 0.008$) for PEG cows when compared to CTR cows (OR = 1.79, 95% CI: 1.16 to 2.76) in the first 30 DIM but were not different ($P = 0.13$) by 60 DIM (OR = 1.32, 95% CI: 0.91 to 1.92). The incidence of SCH was not affected by treatment (13.1 vs 12.1% for PEG vs CTR; $P = 0.88$). Overall, treatment with PEG increased ($P = 0.03$) the incidence of clinical diseases (46.04 vs 35.95% for PEG vs CTR). Cows treated with PEG had higher odds of being diagnosed with 1 or more clinical diseases compared to CTR cows (OR = 1.39, 95% CI: 1.02 to 1.90).

We also evaluated the effect of treatment on the incidences of CM by pathogen and lameness by lesion type (Table 6.3). Treatment with PEG did not affect the incidence of CM by pathogen. However, multiparous cows treated with PEG had a higher ($P = 0.02$) incidence of foot rot when compared with controls (2.46 vs 0.00% for PEG vs CTR). Moreover, multiparous cows treated with PEG tended to have ($P = 0.06$) a higher incidence of white line disease than CTR cows (3.47 vs 6.26% for PEG vs CTR). Furthermore, PEG multiparous cows had ($P = 0.03$) a higher incidence of lameness with no lesion detected when compared with CTR multiparous cows (10.21 vs 4.94% for PEG vs CTR). Additionally, the severity mastitis score did not differ between the PEG and CTR groups (data not shown).

A Poisson regression model was used to evaluate the effect of treatment on the number of mastitis cases during the first 30 d postpartum (PEG = 26; CTR = 18). No differences ($P = 0.16$) were observed (risk ratio = 1.52, 95% CI: 0.84 to 2.73).

A logistic regression model was used to assess the effect of treatment on the odds of experiencing a hospital pen trip within the first 30 d postpartum. No differences ($P = 0.13$) were observed between the PEG and CTR groups (OR = 1.44, 95% CI: 0.90 to 2.29). Lastly, treatment did not alter ($P = 0.66$) the hazard of culling during the first 180 DIM (HR: 0.88, 95% CI: 0.59 to 1.28; Supplementary Figure 6.3).

Effect of Pegbovigrastim on Linear Scores

The effect of PEG on SCS at 7, 14, 21, 28 d relative to calving, and at first, second, and third DHI test is illustrated in Figure 6.6. Treatment with PEG did not affect SCS.

Effect of Pegbovigrastim on Reproductive Performance and Body Condition Score

Treatment did not alter ($P = 0.92$) the hazard of pregnancy during the first 180 DIM (HR: 0.99, 95% CI: 0.84 to 1.7; Supplementary Figure 6.3). The median calving-to-conception interval for PEG and CTR cows was 103 and 104 d, respectively. No differences were observed between the PEG and CTR groups for pregnancy per AI at first service (34.3 vs 28.8% for PEG vs CTR; $P = 0.11$). Moreover, no differences were detected between the PEG and CTR groups on pregnancy loss after first postpartum AI (8.9 vs 11.4% for PEG vs CTR; $P = 0.51$). Furthermore, treatment did not alter ($P = 0.66$) the hazard of being inseminated within the first 120 DIM (HR: 1.03, 95% CI: 0.89 to 1.19; Supplementary Figure 6.3). Body condition score loss from calving until 35 d after parturition was not affected by treatment ($P = 0.88$).

Effect of Pegbovigrastim on Milk Yield and Composition

The effects of PEG treatment on weekly milk averages (first 12 wk of lactation), and ECM, FCM, and milk components for the first 3 months of lactation are presented in Table 6.4. A significant interaction between treatment and parity was detected for milk production ($P \leq 0.03$); primiparous cows treated with PEG produced 1.6 kg/d more milk compared with controls, and no differences were detected between the PEG and CTR groups for multiparous cows. No differences were observed on ECM, FCM, and milk composition between the PEG and CTR groups (Table 6.4).

Daily milk weights for the first 30 DIM were used to evaluate the effect of treatment on cows diagnosed with CM, lameness within the first 30 DIM, metritis, puerperal metritis, and clinical diseases (Table 6.5). No differences were observed between treatment groups for cows

diagnosed with CM or clinical diseases. Among cows diagnosed lame, PEG cows tended to produce more milk ($P = 0.09$) than CTR. Multiparous cows treated with PEG and diagnosed with metritis produced more milk ($P = 0.01$) than CTR.

Effect of Pegbovigrastim on Colostrum Immunoglobulin and Lactoferrin concentrations

Treatment did not affect the concentrations of IgA, IgM, IgG, and lactoferrin in colostrum (Figure 6.7).

Table 6.1. Descriptive data for cows enrolled in the study.

Item	Treatment		*P*-value
	Control (LSM ± SEM)	Pegbovigrastim (LSM ± SEM)	Treatment
Median parity	2	2	-
No. of primiparous, n	120	118	-
No. of multiparous, n	303	299	-
Total, n	423	417	-
BCS at enrollment	3.67 ± 0.01	3.68 ± 0.01	0.85
Rectal temp. at enrollment, °C	38.7 ± 0.01	38.7 ± 0.01	0.80
Dry period of multiparous, d	55.62 ± 0.59	56.01 ± 0.59	0.64
305-ME[1], kg	13901 ± 116	13840 ± 117	0.71
Sire PTA for milk, kg	881 ± 39.64	886 ± 40.01	0.92
Days of gestation, d	277.81 ± 0.22	277.46 ± 0.22	0.24
Twins, %	1.42	1.44	0.98
Male calf, %	48.46	50.36	0.58
Stillbirth, %	8.33	7.63	0.84

[1]305-d mature equivalent lactation yields.

Table 6.2. Incidence of periparturient diseases of cows treated with pegbovigrastim (PEG, n = 417) and controls (CTR, n = 423) by parity (primiparous, PEG, n = 118, CTR, n =120; multiparous, PEG, n = 299, CTR, n = 303). Outcomes from mixed logistic regression models are presented; control was considered as the reference group.

	Incidence (%)						OR[1] (95% CI)[2]	*P*-value[3]		
	Controls			Pegbovigrastim				Trt[4]	Parity	Trt × Parity
	All cows	Primip.	Multip.	All cows	Primip.	Multip.				
Disease										
Stillbirth	4.73	8.33	3.30	3.84	7.63	2.34	0.80 (0.40, 1.57)	0.52	<0.01	0.71
Dystocia	4.02	9.17	1.98	5.76	11.02	3.68	1.47 (0.77, 1.57)	0.24	<0.01	0.52
Retained placenta	4.49	4.17	4.62	4.08	3.39	4.35	0.90 (0.46, 1.76)	0.76	<0.01	0.19
Metritis	11.98	20.51	8.56	14.29	21.24	11.60	1.24 (0.82, 1.88)	0.30	<0.01	0.46
Puerperal metritis	15.37	30.83	9.24	17.51	35.59	10.37	1.15 (0.78, 1.70)	0.46	<0.01	0.80
Metritis-PM	20.80	36.67	14.52	25.18	39.83	19.40	1.27 (0.91, 1.77)	0.16	<0.01	0.54
Fever	7.18	11.11	5.57	10.97	20.35	7.29	1.48 (0.88, 2.48)	0.14	<0.01	0.36
Hyperketonemia[5]	21.43	20.51	22.03	19.59	5.56	27.87	0.23 (0.04, 1.18)	0.23	0.04	0.05
Displaced abomasum	0.24	0.00	0.33	1.92	0.85	2.34	8.27 (1.02, 66.6)	0.05	0.27	-[9]
Clinical mastitis	4.26	1.67	5.28	6.24	5.08	6.69	1.51 (0.81, 2.80)	0.18	0.14	0.33
SCM, day 7[6]	24.75	20.00	25.93	23.53	30.43	21.52	0.93 (0.49, 1.78)	0.84	0.87	0.33
SCM, day 10[7]	16.94	20.00	15.71	19.15	25.74	16.54	0.97 (0.58, 1.61)	0.97	0.07	0.41
SCM, day 14[6]	15.24	11.11	16.09	15.53	20.00	14.46	0.84 (0.36, 1.98)	0.70	0.97	0.43
SCM, day 21[6]	15.96	5.56	18.42	18.95	15.79	19.74	1.23 (0.57, 2.62)	0.59	0.20	0.40
SCM, day 28[6]	11.83	10.00	12.33	16.30	4.55	20.00	1.48 (0.63, 3.47)	0.36	0.16	0.29
SCM, 1st DHI	19.84	22.52	18.68	23.58	24.75	23.11	1.24 (0.89, 1.93)	0.34	0.54	0.82
SCM, 2nd DHI	12.04	8.04	13.88	12.54	9.82	13.81	0.94 (0.55, 1.60)	0.83	0.05	0.53
Lameness	9.68	3.39	12.28	15.69	3.39	20.69	1.79 (1.16, 2.76)	<0.01	<0.01	0.41
Endometritis	12.85	19.13	10.28	13.16	15.52	12.19	1.02 (0.67, 1.55)	0.90	0.02	0.31
Clinical diseases[8]	35.93	45.83	32.01	46.04	50.00	44.48	1.39 (1.02, 1.90)	0.03	0.04	0.23

Primp. = Primiparous.
Multip. = Multiparous.
Metritis-PM = Metritis and puerperal metritis combined.
HYK = Hyperketonemia.
SCM = Subclinical Mastitis.

DHI = Dairy Herd Improvement Association.
[1]Odds ratio.
[2]95% confidence interval.
[3]Variables with *p*-values > 0.15 were forced into the model to report *p*-value.
[4]Trt, treatment.
[5]The incidence of hyperketonemia was evaluated from the subset cows from which blood was collected (primiparous: PEG, n = 37, CTR, n = 39; multiparous: PEG, n = 65, CTR, n = 64).
[6]Sublinical mastitis at d 7, 14, 21 and 28 was evaluated from a subset of cows (PEG, n = 102; 37 primiparous, 65 multiparous; CTR, n = 103; 39 primiparous, 64 multiparous).
[7]Sublinical mastitis at d 10 was evaluated in all cows enrolled in the study, by using a California Mastitis Test after milking and the results were recorded for each quarter of the udder.
[8]The following clinical diseases were used to evaluate the effect of treatment on the proportion of cows affected by clinical diseases: retained placenta, metritis, puerperal metritis, endometritis, displaced abomasum, lameness, and clinical mastitis.
[9]*P*-value of the interaction term treatment by parity is not reported due to non-observed cases of displaced abomasum in CTR primiparous cows.

Table 6.3. Incidence of clinical mastitis by pathogen and lameness by lesion type during the first 30 days after calving of cows treated with pegbovigrastim (PEG, n = 417) and controls (CTR, n = 423) by parity (primiparous: PEG, n = 118; CTR, n = 120; multiparous: PEG, n = 299; CTR, n = 303).

	Incidence								
	%						*Chi-Square Test*		
	Controls			Pegbovigrastim			*P*-value		
	All cows	Primip.	Multip.	All cows	Primp.	Multip.	All cows	Primp.	Multip.
Mastitis pathogen									
Escherichia coli	0.25	0.00	0.34	0.76	0.88	0.71	0.36	0.48	0.62
Klebsiella sp.	1.22	0.84	1.37	1.51	0.00	2.11	0.72	1.00	0.49
Streptococcus sp.	0.74	0.00	1.03	2.01	2.61	1.76	0.12	0.12	0.45
No Growth	2.17	0.84	2.71	2.25	1.75	2.45	0.94	0.53	0.84
Lameness lesion type									
Foot rot	0.00	0.00	0.00	1.61	0.00	2.46	0.02	-	0.02
Sole ulcer	3.19	0.00	4.58	1.17	0.00	2.54	0.20	-	0.22
White line disease	2.41	0.00	3.47	4.97	0.00	6.26	0.09	-	0.06
No lesion detected[1]	4.46	3.39	4.94	8.02	3.39	10.21	0.06	1.00	0.03
Others	0.27	0.00	0.40	1.43	0.00	2.13	0.12	-	0.11

Primp. = Primiparous.
Multi. = Multiparous.
[1]No lesion detected was defined as cows being diagnosed lame with no observed macroscopic lesion in the feet.

Table 6.4. Weekly milk yield for the first 12 wk after calving, and ECM, FCM, and milk composition for the first three months of lactation of cows treated with pegbovigrastim (PEG, n = 417) and controls (CTR, n = 423). Results are presented as LSM ± SEM.

Item	Primiparous		Multiparous		*P*-value				
	CTR (LSM ± SEM)	PEG (LSM ± SEM)	CTR (LSM ± SEM)	PEG (LSM ± SEM)	Trt[1]	Parity	Time	Trt × Parity	Trt × Time
Milk yield (kg/d), first 12 weeks	35.91±0.65[a]	37.51±0.66[b]	49.67±0.42	48.89±0.42	0.46	<0.001	<0.001	0.03	0.25
Energy corrected milk (kg/d)	37.19±0.72	37.32±0.72	52.68±0.47	51.72±0.47	0.26	<0.001	<0.001	0.37	0.55
3.5% Fat corrected milk (kg/d)	36.44±0.74	36.86±0.74	51.92±0.47	51.03±0.48	0.71	<0.001	<0.001	0.29	0.66
Fat, kg[2]	1.34±0.03	1.33±0.03	1.92±0.02	1.89±0.02	0.53	0.001	<0.001	0.60	0.67
Protein, kg[2]	1.12±0.02	1.11±0.02	1.58±0.01	1.54±0.01	0.20	<0.001	<0.001	0.47	0.76
Fat (%)[2]	4.04±0.05	3.93±0.05	4.12±0.03	4.12±0.03	0.23	0.001	<0.001	0.23	0.94
Protein (%)[2]	3.15±0.03	3.09±0.03	3.11±0.02	3.09±0.02	0.09	0.24	<0.001	0.36	0.81

[1]Trt, treatment.

[a-b]Different superscripts within a row indicate a significant difference ($P \leq 0.05$).

[2]Fat and protein were recorded by Dairy Herd Improvement Association (DHI) for the first 3 months of lactation.

Table 6.5. Milk yield for the first 30 d in milk (kg/d) of cows treated with pegbovigrastim (PEG) and controls (CTR) diagnosed with clinical mastitis, lame in the first 30 DIM, metritis, puerperal metritis, metritis-PM, and clinical diseases.

Item	n		Milk yield (kg/d), first 30 d in milk		
	CTR	PEG	CTR (LSM ±SEM)	PEG (LSM ± SEM)	*P*-value
Clinical mastitis					
All cows[1]	16	26	32.50 ± 2.43	33.67 ± 1.84	0.66
Lameness					
All cows[1]	39	64	34.91 ± 1.24	36.94 ± 1.17	0.09
Metritis					
Primiparous	24	24	27.85 ± 0.87	28.50 ± 0.88	0.59
Multiparous	25	34	37.61 ± 1.26	41.78 ± 1.06	0.01
Puerperal metritis					
Primiparous	37	42	28.04 ± 0.85	29.95 ± 0.81	0.50
Multiparous	28	31	40.82 ± 0.98	41.13 ± 0.94	0.82
Metritis-PM					
Primiparous	44	47	28.33 ± 0.54	29.20 ± 0.54	0.65
Multiparous	44	58	40.40 ± 0.70	41.46 ± 0.61	0.67
Clinical diseases					
Primiparous	55	59	25.57 ± 0.73	26.48 ± 0.71	0.37
Multiparous	97	133	38.55 ± 0.55	38.93 ± 0.47	0.60

[1]Milk yield was not evaluated by parity due to small sample size.
Metritis-PM = Metritis and puerperal metritis combined.

Figure 6.1. Days from the first to second administration of pegbovigrastim in all treated cows (A; mean = 8.83; range = 1 to 26 days; SD = 4.62; median = 9), treated primiparous cows (B; mean = 8.62; range = 1 to 19 days; SD = 4.29; median = 8), and treated multiparous cows (C; mean = 8.92; range = 1 to 26 days; SD = 4.75; median = 9).

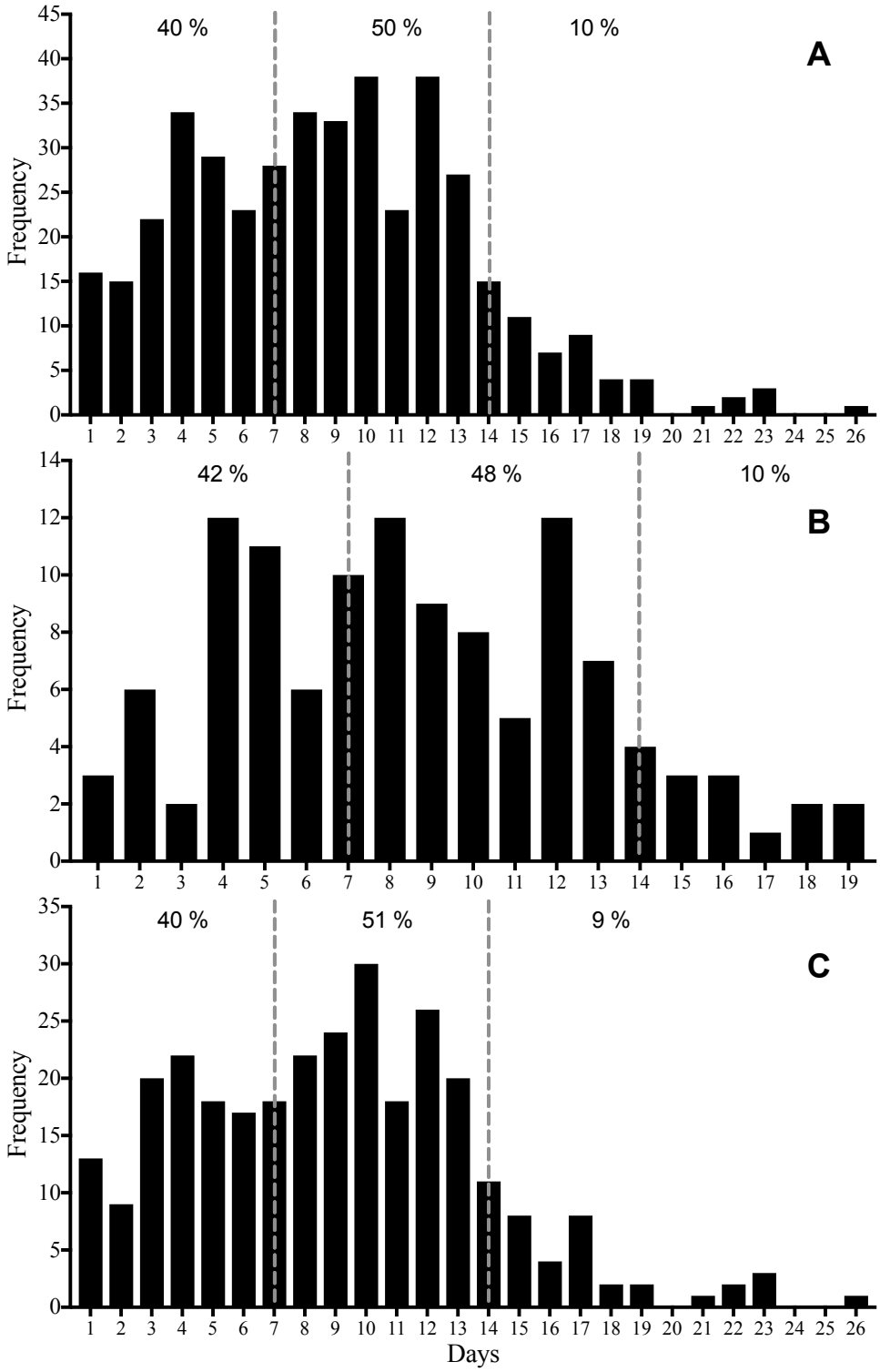


Figure 6.2. White blood cell (A), lymphocyte (B), PMN (C), and monocyte (D) concentrations of cows treated with pegbovigrastim (PEG, n = 102) and control (CTR, n = 103) cows at -7, 0, 3, 7 and 14 d relative to parturition. Results are presented as LSM ± SEM. *$P \leq 0.001$.

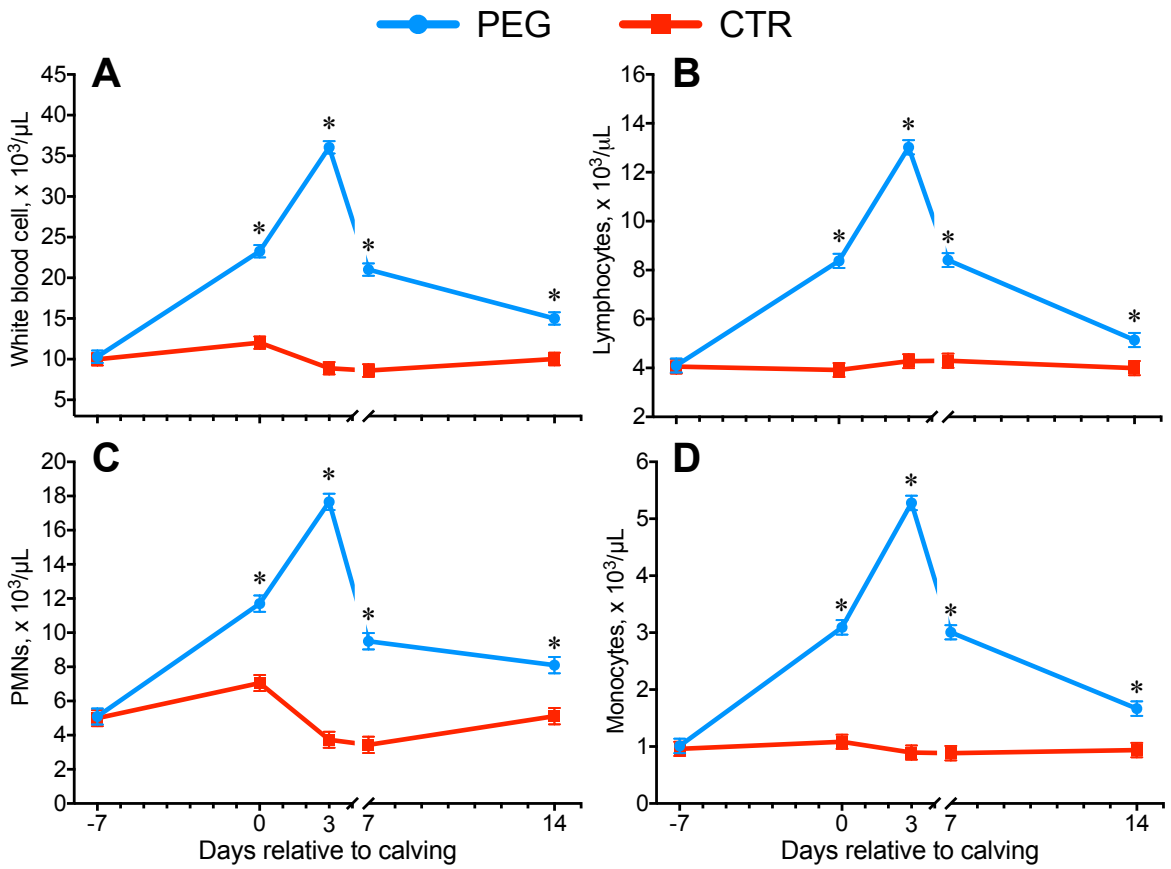


Figure 6.3. White blood cell, PMN, lymphocyte, and monocyte concentrations of metritic (panel A), mastitic (panel B), and pregnant and open cows at 120 DIM (panel C) of a subset cows treated with pegbovigrastim (PEG, n = 102) and controls (CTR, n = 103) at -7, 0, 3, 7 and 14 d relative to parturition. Results are presented as LSM ± SEM. • Represents the comparison of PEG vs CTR cows diagnosed with metritis and/or puerperal metritis (metritis-PM), mastitis or pregnant at 120 DIM. ° Represents the comparison of PEG vs CTR cows without metritis-PM or mastitis, or cows diagnosed open at 120 DIM. ◆ Represents the comparison of PEG cows diagnosed with metritis-PM or mastitis vs PEG non-metritic or non-mastitic cows, or PEG pregnant cows vs PEG open cows at 120 DIM. * $P \leq 0.05$, † $P \leq 0.1$.

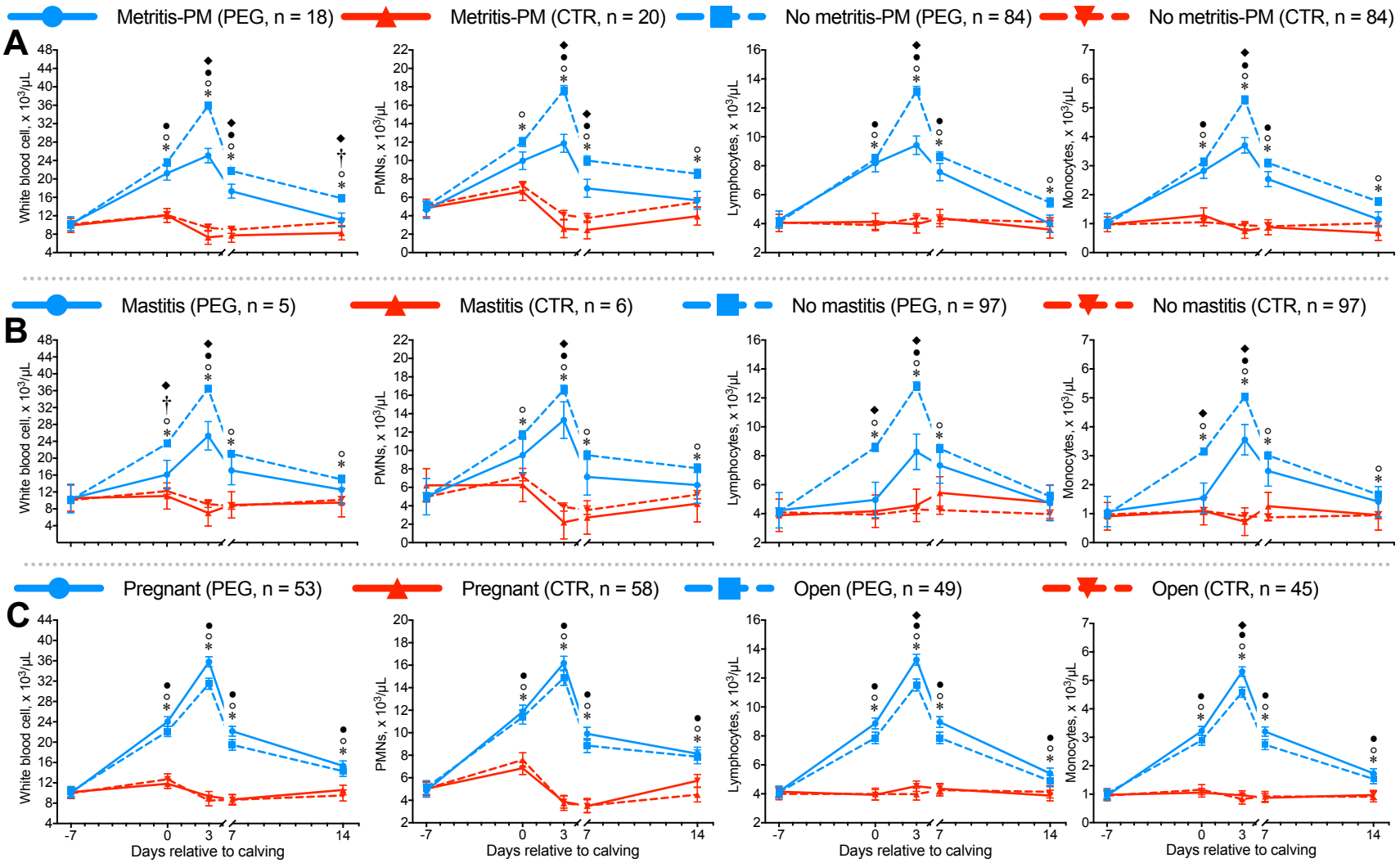


Figure 6.4. Plasma concentrations of non-esterified fatty acids (NEFA), β-hydrohybutyrate (BHB), and haptoglobin (optical density units) of cows treated with pegbovigrastim (PEG, n = 102) and controls (CTR, n = 103) at -7, and 0, 3, 7 and 14 d relative to parturition. Results are presented as LSM ± SEM.

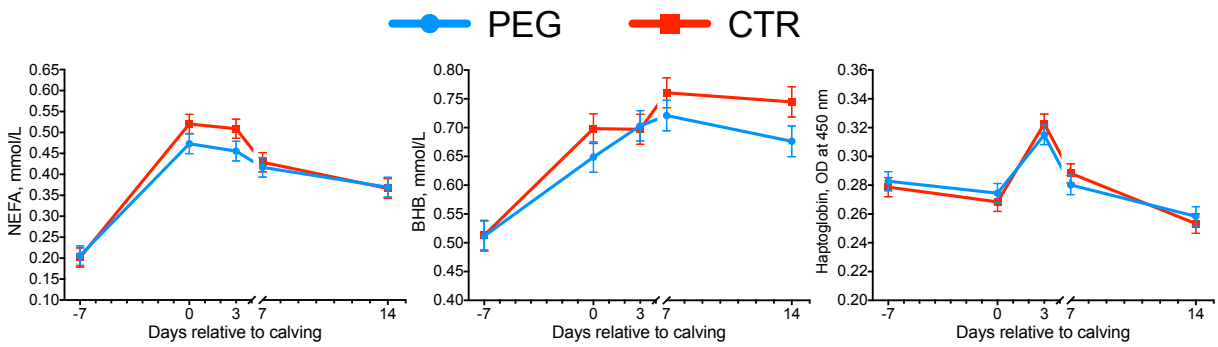


Figure 6.5. Percentage of neutrophils identified in the vagina by cytobrush for cows treated with pegbovigrastim (PEG, n = 102) and controls (CTR, n = 103) at 0, 7 and 35 d relative to parturition (A). Percentage of neutrophils of PEG and CTR cows diagnosed with metritis and/or puerperal metritis (metritis-PM) at 0, 7 and 35 d relative to parturition (B). Results are presented as LSM ± SEM.* $P \leq 0.05$.

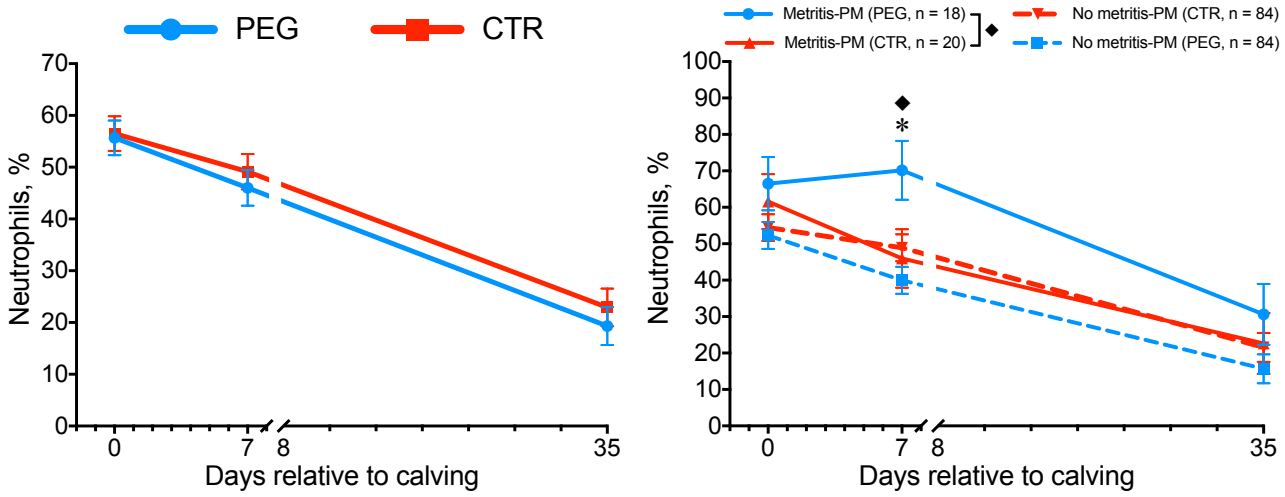


Figure 6.6. Linear scores of cows treated with pegbovigrastim (PEG) and controls (CTR) at 7, 14, 21, and 28 d relative to parturition [left side of the dotted line (PEG, n = 110; CTR, n = 119)], and at first, second, and third DHI test [right side of the dotted line (PEG, n = 417; CTR, n = 423)]. Results are presented as LSM ± SEM.

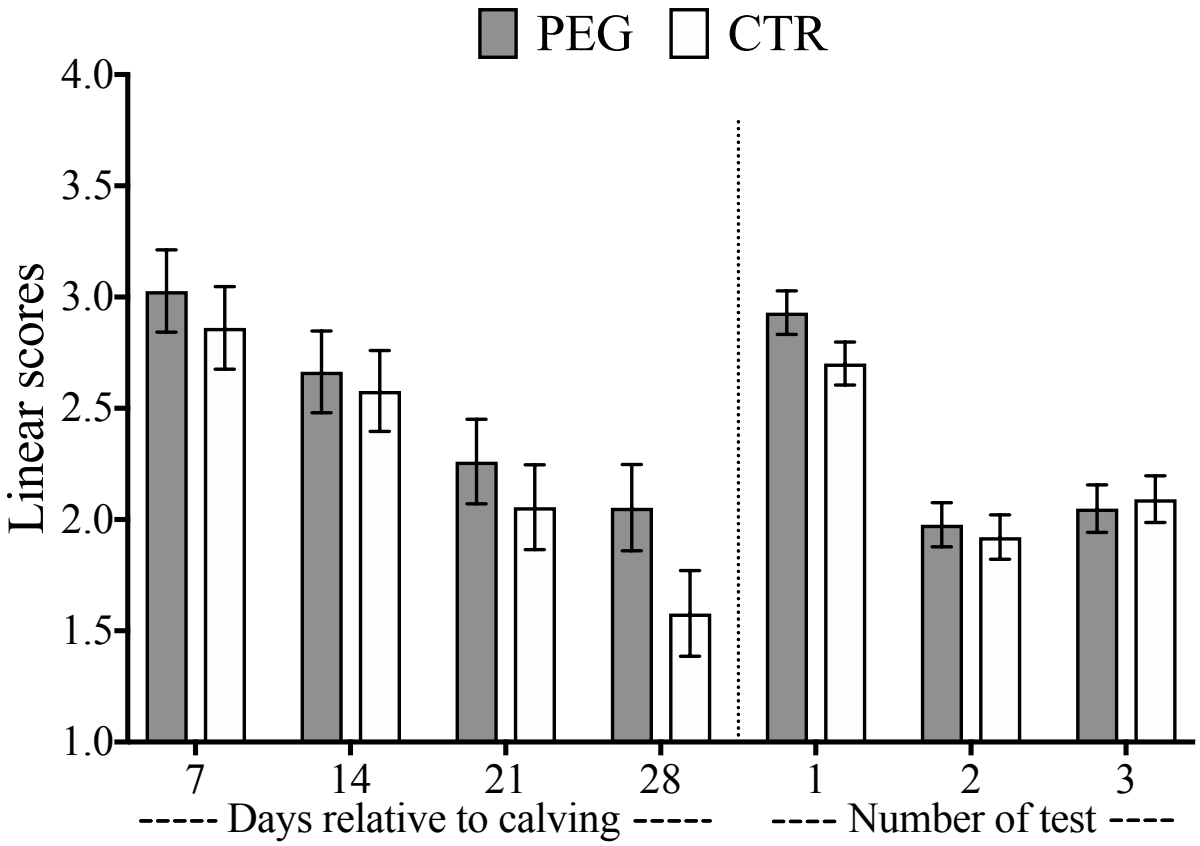
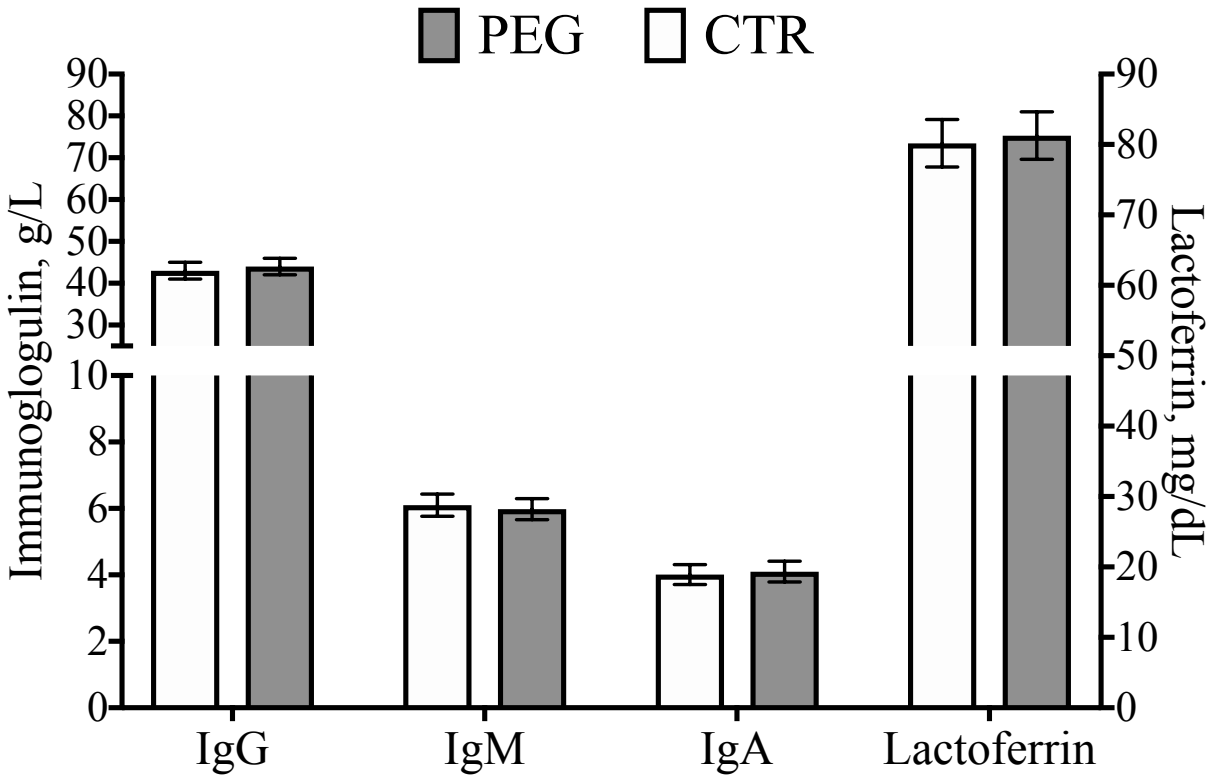


Figure 6.7. Colostrum concentrations of immunoglobulin (Ig) G, M, and A, and lactoferrin of cows treated with pegbovigrastim (PEG, n = 100) and controls (CTR, n = 100). Results are presented as LSM ± SEM.



SUPLEMENTARY INFORMATION

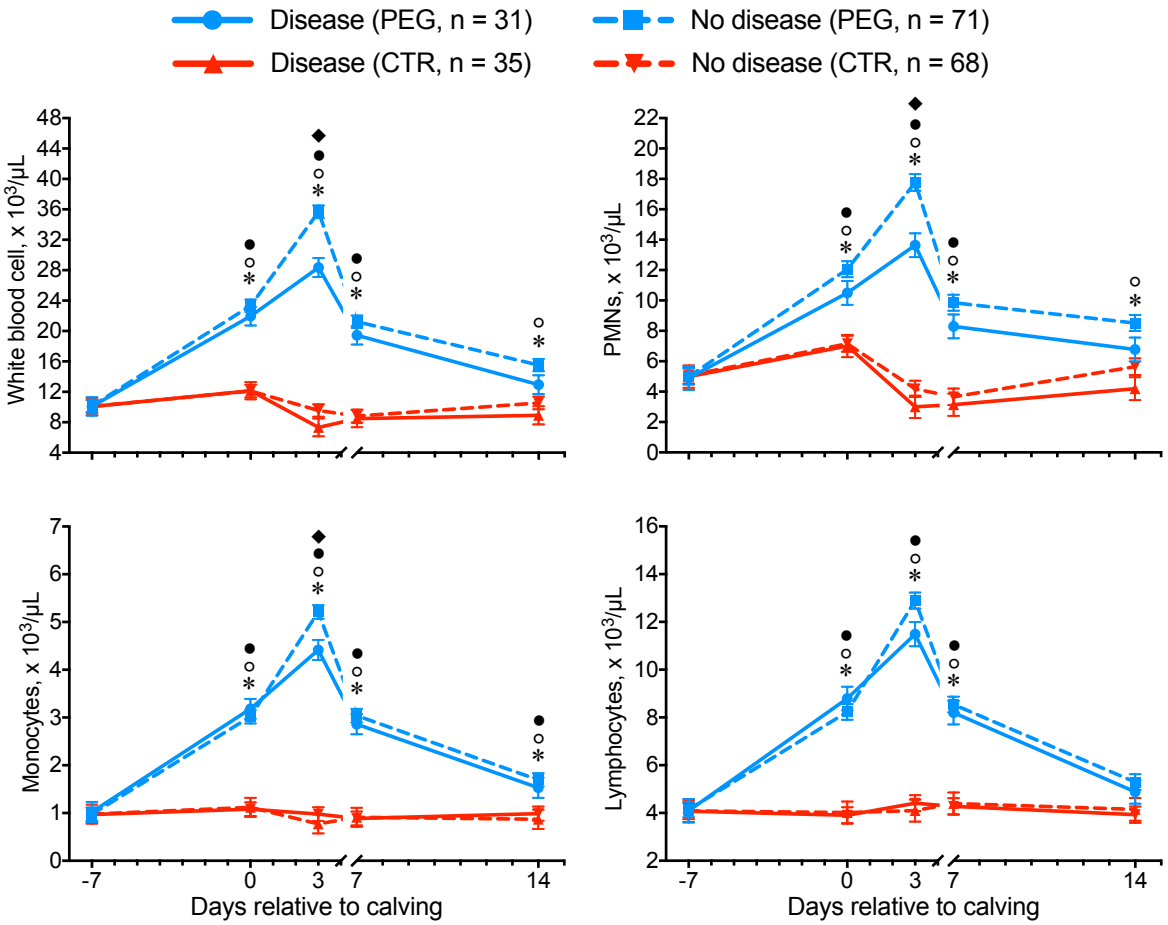
Supplementary Table 6.1. White blood cell (WBC), lymphocyte (LYM), PMN, and monocyte concentrations of cows treated with pegbovigrastim and controls diagnosed with metritis and/or puerperal metritis (metritis-PM), mastitis, clinical diseases, and pregnant and open cows at 120 d in milk at -7, 0, 3, 7 and 14 d relative to parturition. Results are presented as LSM ± SEM.

	Control		Pegbovigrastim		*P*-value				
Event	No event (LSM ± SEM)	Event (LSM ± SEM)	No event (LSM ± SEM)	Event (LSM ± SEM)	Trt	Parity	Time	Trt × Event	Trt × Event × Time
Metritis-PM									
WBC, × 10^3/μL	10.25±0.41	9.05±0.81	21.43±0.41	16.99±0.82	<0.001	0.01	<0.001	<0.001	0.001
LYM, × 10^3/μL	4.16±0.16	4.02±0.33	7.97±0.16	6.68±0.33	<0.001	0.30	<0.001	0.002	0.002
PMN, × 10^3/μL	5.13±0.25	4.09±0.50	10.65±0.25	7.83±0.50	<0.001	0.02	0.003	<0.001	0.05
Monocytes, × 10^3/μL	0.98±0.07	0.91±0.14	2.84±0.07	2.26±0.14	<0.001	0.10	<0.001	0.001	0.004
Mastitis									
WBC, × 10^3/μL	10.03±0.39	9.38±1.57	20.75±0.39	16.28±1.69	<0.001	0.03	<0.001	0.03	0.57
LYM, × 10^3/μL	4.10±0.15	4.57±0.61	7.81±0.15	5.90±0.66	<0.001	0.45	<0.001	0.01	0.15
PMN, × 10^3/μL	4.96±0.23	4.30±0.93	9.97±0.23	8.25±0.99	<0.001	0.02	<0.001	0.19	0.94
Monocytes, × 10^3/μL	0.96±0.06	0.99±0.26	2.77±0.07	2.01±0.28	<0.001	0.15	<0.001	0.03	0.27
Clinical diseases									
WBC, × 10^3/μL	10.21±0.45	9.38±0.62	21.18±0.44	18.55±0.67	<0.001	0.05	<0.001	0.002	0.01
LYM, × 10^3/μL	4.11±0.18	4.15±0.25	7.82±0.18	7.49±0.20	<0.001	0.33	<0.001	0.60	0.41
PMN, × 10^3/μL	5.14±0.28	4.46±0.39	10.63±0.27	8.81±0.41	<0.001	0.05	0.001	0.006	0.14
Monocytes, × 10^3/μL	0.98±0.08	0.93±0.10	2.78±0.07	2.60±0.11	<0.001	0.14	<0.001	0.38	0.18
Pregnancy at 120 DIM									
WBC, × 10^3/μL	9.86±0.56	10.18±0.59	19.82±0.58	22.10±0.57	<0.001	0.06	<0.001	0.04	0.48
LYM, × 10^3/μL	4.07±0.23	4.16±0.20	7.24±0.22	8.12±0.21	<0.001	0.68	<0.001	0.01	0.53
PMN, × 10^3/μL	4.76±0.31	4.97±0.39	9.61±0.31	11.20±0.40	<0.001	0.03	<0.001	0.44	0.74
Monocytes, × 10^3/μL	0.94±0.08	0.98±0.09	2.55±0.09	2.90±0.09	<0.001	0.28	<0.001	0.04	0.50

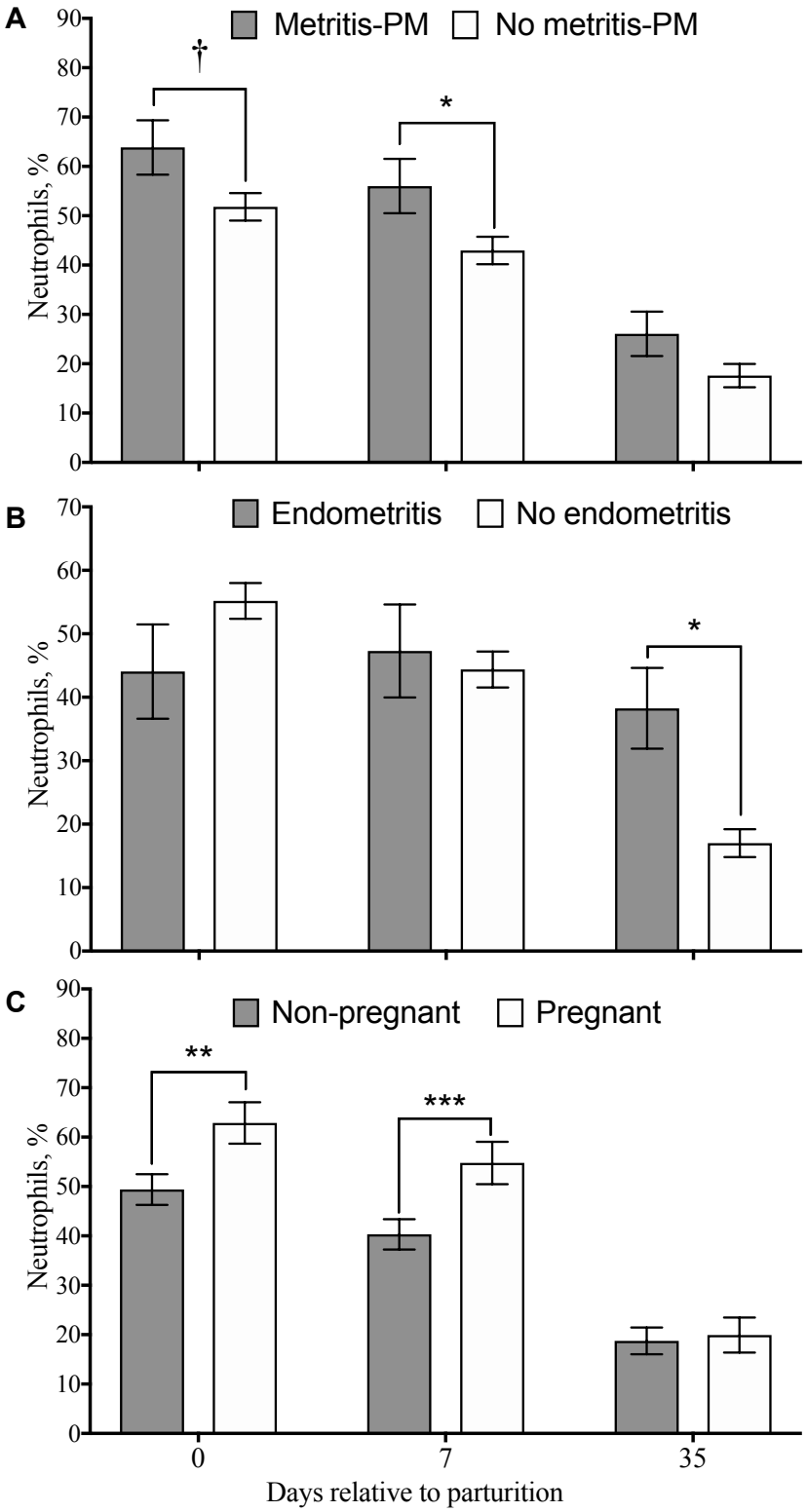
Supplementary Table 6.2. Plasma mineral concentration at the day of parturition (after calving) of cows (≥ 3 parity) treated with pegbovigrastim (PEG, n = 33) and controls (CTR, n = 30). Results are presented as LSM ± SEM.

Item	Multiparous (≥ 3 parity)		*P*-value
	CTR (LSM ± SEM)	PEG (LSM ± SEM)	Treatment
Calcium, mmol/L	2.19±0.06	2.27±0.05	0.31
Copper, mmol/L	0.01±0.0004	0.02±0.0003	0.15
Iron, mmol/L	0.03±0.003	0.04±0.004	0.47
Potassium, mmol/L	20.52±0.65	20.61±0.65	0.93
Magnesium, mmol/L	0.84±0.02	0.83±0.02	0.73
Manganese, mg/L	0.001±0.0001	0.001±0.0001	0.46
Phosphorous, mmol/L	0.95±0.04	0.94±0.04	0.90
Selenium, mmol/L	0.001±0.00002	0.001±0.00002	0.54
Zinc, mmol/L	0.001±0.0004	0.001±0.0004	0.76

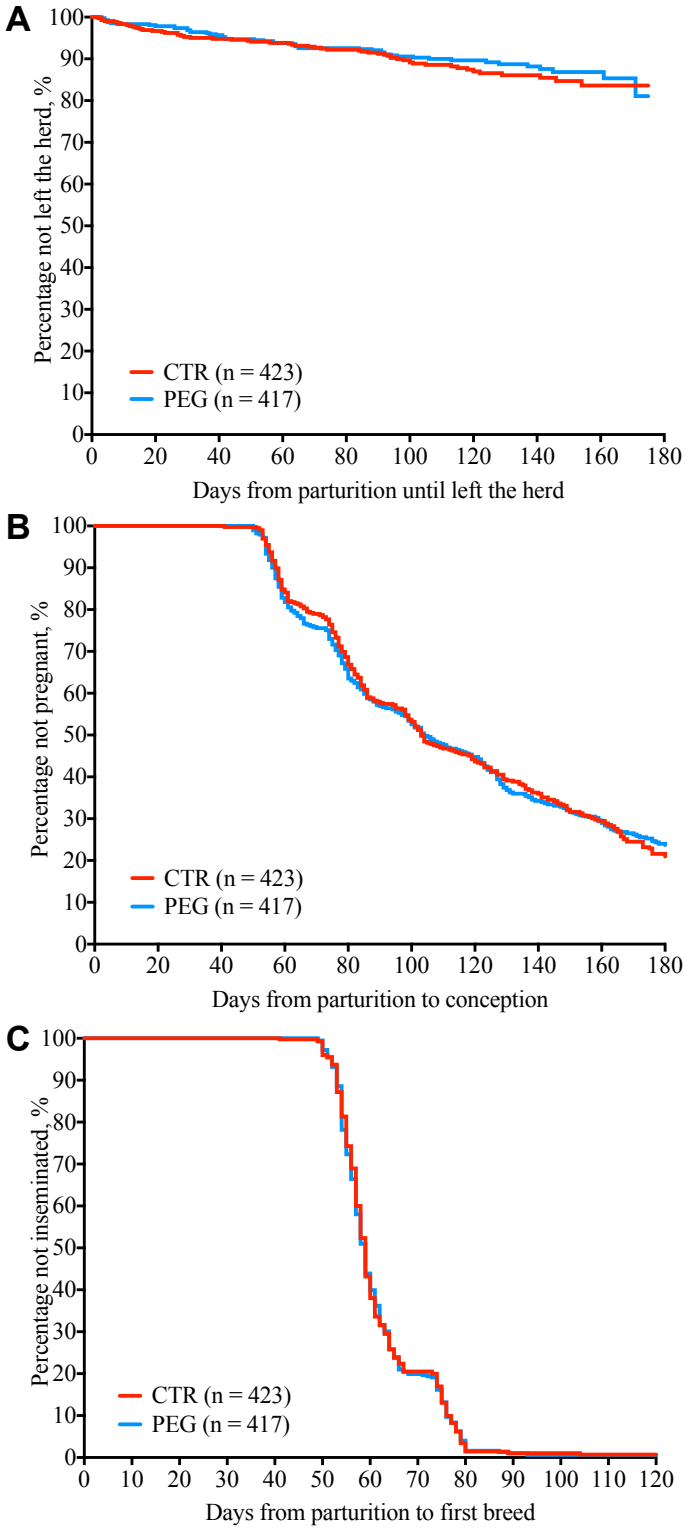
Supplementary Figure 6.1. White blood cell, PMN, lymphocyte, and monocyte concentrations of cows diagnosed with 1 or more clinical disease of a subset cows treated with pegbovigrastim (PEG, n = 102) and controls (CTR, n = 103) at -7, 0, 3, 7 and 14 d relative to parturition. Results are presented as LSM ± SEM. • Represents the comparison of PEG vs CTR cows diagnosed with clinical disease. ° Represents the comparison of PEG vs CTR cows with no clinical disease. ◆ Represents the comparison of PEG cows diagnosed clinical disease vs PEG cows with no clinical disease. * $P \leq 0.05$, † $P \leq 0.1$.



Supplementary Figure 6.2. Percentage of neutrophils identified in the vagina by cytobrush of cows diagnosed with metritis and/or puerperal metritis (metritis-PM) and no metritis-PM cows (panel A), endometritis and no endometritis cows (panel B), and pregnant and non-pregnant cows at 120 DIM (panel C) at 0, 7, and 35 d relative to parturition. Error bars are SEM. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, † $P \leq 0.1$.



Supplementary Figure 6.3. (A) Kaplan-Meier survival curves of calving to left the herd in days of cows treated with pegbovigrastim (PEG) and controls (CTR). No differences ($P = 0.48$) were observed between PEG and CTR groups (HR: 0.87, 95% CI: 0.59 to 1.28). (B) Kaplan-Meier survival curves of calving to conception interval in days of PEG and CTR cows. No differences ($P = 0.92$) were observed between PEG and CTR groups (HR: 0.99, 95% CI: 0.84 to 1.17). (C) Kaplan-Meier survival curves of calving to first service in days of PEG and CTR cows. No differences ($P = 0.66$) were observed between PEG and CTR groups (HR: 1.03, 95% CI: 0.89 to 1.19).



DISCUSSION

In support of our initial hypothesis, cows treated with PEG exhibited a 3- to 4-fold increase in circulating WBC compared with CTR cows. However, treatment did not alter the incidence of mastitis (clinical or subclinical), uterine diseases, and did not affect reproductive performance. Cows treated with PEG were more likely to be diagnosed with 1 or more of the evaluated clinical diseases when compared with CTR cows. Additionally, we observed that primiparous cows treated with PEG produced more milk during the first 12 wk after parturition than CTR primiparous cows. However, PEG did not affect ECM, FCM, and milk components. The present study identified, for the first time, a detrimental effect of PEG treatment on postpartum lameness and DA.

Polymorphonuclear cells are the main cell line of defense involved in bacterial clearance after uterine (Hussain, 1989) and mammary gland (Paape et al., 2002) infection, and play a major role in placental release (Kimura et al., 2002). However, peripheral blood PMN function of periparturient dairy cows is impaired relative to non-parturient cows (Kehrli et al., 1989; Cai et al., 1994). Moreover, blood neutrophil function begins to decline prior to parturition, and slowly returns to prepartum levels approximately 4 wk postpartum (Kehrli et al., 1989; Goff and Horst, 1997). Thus, increasing WBC in periparturient dairy cows could be a good strategy to mitigate the immunosuppression observed during the transition from late gestation through early lactation.

Our results are in accordance with previous studies showing that PEG treatment increases circulating WBC (Kimura et al., 2014; Canning et al., 2017; McDougall et al., 2017). More specifically, our finding of increased blood PMNs agrees with the literature; however, discrepant results have been reported for MONO and LYM concentrations (Kimura et al., 2014; McDougall et al., 2017). Kimura et al. (2014) observed no differences between PEG-treated and untreated cows in terms of MONO and LYM numbers. In contrast, a study by McDougall et al. (2017) reported a significant increase in MONO and LYM counts. Moreover, similar increases in MONO and LYM concentrations were observed in humans treated with G-CSF (Pollmacher et

al., 1996; Reyes et al., 1999). Based on our results and those reported by McDougall et al. (2017), we conclude that PEG treatment increases PMN, MONO, and LYM numbers in Holstein cows.

Neutrophils are the first line of defense to arrive in the mammary gland once pathogen invasion occurs. Therefore the ability of the mammary gland to counteract bacterial infection relies mainly on: (1) circulating neutrophils and their ability to enter the alveoli lumen, and (2) their phagocytic and killing capacity (Ezzat et al., 2014). The peripartum administration of PEG has been associated with a decreased incidence of CM in recent studies (Hassfurther et al., 2015; Canning et al., 2017; Ruiz et al., 2017). However, in the present study, the incidence of CM and SCM did not differ between the PEG and CTR groups. In the studies conducted by Canning et al. (2017) and Hassfurther et al. (2015) the incidence of CM in the first 30 DIM for control cows was 23 and 34%, respectively. Herein, the incidence of CM in the first 30 DIM for the CTR group was much lower (4.26%). Thus, it is possible that the lack of a preventive effect of PEG on postpartum CM in our study was due to the very low incidence of CM during the first 30 DIM. However, in the study conducted by Ruiz et al. (2017) the incidence of CM was about 4.9% and the administration of PEG significantly reduced the incidence of CM. Nevertheless, in the present study, the incidence of CM was numerically higher in the PEG group when compared with CTR group and no effect of treatment on SCS was identified. In support of our findings, Canning et al (2017) reported no effect of PEG treatment on SCC.

It is also possible that the key to prevent the occurrence of CM during the early postpartum period would be to increase the phagocytic and killing ability of neutrophils and not simply increasing their counts in blood. In support to this theory, recent studies have been shown that neutrophil phagocytic activity, oxidative burst, and MOP function is not affected by PEG treatment (Kimura et al., 2014; McDougall et al., 2017).

The relationship between uterine diseases and neutrophil function has been the topic of several studies (Gunnink, 1984a; b; Kimura et al., 2002). Kimura et al. (2002) demonstrated that the neutrophils of cows affected with RP had impaired neutrophil function and these differences

were identified as early as 10 d before parturition. A recent study evaluated the proportion of neutrophils in the uterine lumen during the early postpartum and its associations with uterine bacterial infection and reproductive performance (Gilbert and Santos, 2016). Cows with the greatest influx of neutrophils into the uterus in the immediate postpartum period were associated with improved uterine health and reproductive performance. In agreement with the published literature, our study also identified that early influx of neutrophils into the reproductive tract mucosa was associated with increased reproductive performance. However, PEG administration did not induce an early influx of neutrophils into the reproductive tract, and did not improve uterine health and reproductive performance. Curiously, it was observed that the relative counts of neutrophils of cows affected with metritis-PM that were previously treated with PEG was significantly higher at d 7 when compared with CTR cows also affected with metritis-PM. Therefore, the higher proportion of neutrophils observed in PEG-treated cows with metritis-PM may be a result of blood neutrophils migrating into the uterine lumen in response to activated chemokine signaling due to bacterial infection.

In our study, the incidence of metritis and puerperal metritis were not affected by PEG. Conversely, Ruiz et al. (2017) observed a 17% increase in metritis incidence with PEG treatment. In that study, cows affected with metritis were recognized by abnormal uterine discharge (smelly and watery) without (mild metritis) or with (severe metritis) the presence of systemic clinical signs such us fever, depression or lack of appetite within 21 d postpartum. In our study, the criteria used to define a cow affected with metritis-PM (watery and smelly red-brown uterine discharge with or without systemic illness) is comparable to the one used in the study conducted by Ruiz et al. (2017). Similarly, in our study we observed that cows treated with PEG had a numerical increase ($P = 0.16$) of 20% in metritis-PM incidence when compared with CTR cows. Herein, our sample size was not large enough to detect significant differences in metritis-PM incidence; however, our results are in alignment with the results reported by Ruiz et al (2017). It was suggested that a more robust uterine inflammatory response, triggered by a rise in neutrophil numbers and functions, could be the reason of more cows exhibiting clinical

metritis, and that future studies were needed to characterize the PMN cells present in the uterus of cows affected with metritis (Ruiz et al., 2017). In support to that notion, we observed that cows diagnosed with metritis-PM and treated with PEG had higher relative counts of neutrophils in the reproductive tract mucosa when compared with CTR cows with metritis-PM.

Furthermore, we observed that multiparous cows diagnosed with metritis and treated with PEG produced more milk than CTR multiparous cows with metritis. These findings are in alignment with the study conducted by Ruiz et al., (2017). In that study, multiparous cows diagnosed with metritis and treated with PEG showed a higher milk yield than untreated controls. However, in the current study, when milk yield was evaluated between treatment groups within cows diagnosed with metritis-PM, the administration of PEG did not alter milk yield. They also reported that PEG-treated multiparous cows that developed CM tended to produce more milk than CTR mastitic cows. Nevertheless, in the present study, milk yield was not affected by PEG when cows experienced an event of CM.

Interestingly, we found that cows treated with PEG had higher odds of being diagnosed with 1 or more of the evaluated clinical diseases compared with CTR. Based on the detected increase of neutrophils in the vaginal mucosa of PEG cows with metritis-PM compared with CTR cows with metritis-PM, and the higher blood PMNs in diseases treated cows than diseases CTR cows, it is possible that cows treated with PEG and experiencing a bacterial infection might have an increased migration of neutrophils to the site of infection which could lead to exacerbated clinical signs. Therefore, the administration of PEG might trigger a more robust inflammatory response in sick animals and thus more animals exhibiting apparent clinical signs. This hypothesis needs to be further investigated.

In the study by Ruiz et al. (2017), cows that received PEG had a 5.8% higher chance of being inseminated within the first 100 DIM postpartum, and Canning et al. (2017) reported a 52% reduction in failure to return to estrus by 80 DIM in cows treated with PEG, relative to control cows. In our study, reproductive performance was not affected by PEG treatment. Although P/AI at first service was not statistically increased ($P = 0.11$), we observed that cows

treated with PEG had a 5.5-percentage-point numerical increase in P/AI at first service when compared with CTR cows (PEG = 34.3, CTR = 28.8%). The lack of differences in P/AI at first service should be interpreted with caution because this study was not powered to detect significant differences in P/AI between treatment groups with the observed percentage differences. More studies with a larger sample size are needed to evaluate whether differences in P/AI at first service should be expected with the use of PEG.

As an adaptive response to negative energy balance, postpartum dairy cows exhibit an elevation of plasma NEFA and BHB, which are metabolic changes necessary to support milk synthesis and body energy demands (Bauman and Currie, 1980; Baumgard et al., 2017). However, excessive production of BHB during early lactation has been associated with: increased risk of postpartum disorders; decreased milk production; and poor reproductive performance (Duffield et al., 2009; McArt et al., 2012; McArt et al., 2013). In the present study, we observed that primiparous cows treated with PEG tended to have lower odds of developing HYK compared with non-treated primiparous cows. These results should be interpreted with caution since the incidence of HYK was calculated from a small number of cows (PEG, n = 37; CTR, n = 39). Further studies with a larger sample size are needed to evaluate whether differences in HYK during the early postpartum should be expected with the use of PEG, and to elucidate the mechanism by which PEG lowers the incidence of HYK. Additionally, we found no association between NEFA and PEG administration, which is in agreement with previous work (McDougall et al., 2017).

Canning et al. (2017) reported similar milk composition between PEG-treated and control cows. Our results presented here on milk composition confirm that PEG does not affect protein and fat synthesis. We did observe that primiparous animals treated with PEG produced more milk when compared with CTR primiparous cows; however, ECM yield was not affect by PEG. Assuming that DMI was similar between treatment groups, the underlying mechanism that primiparous cows treated with PEG faced to produce more milk warrants further investigation.

Moreover, our finding that administration of PEG 7 d before the anticipated date of calving does not affect calf viability is also in agreement with the study by Canning et al. (2017).

We did observe that animals treated with PEG had a higher incidence of DA relative to controls. The development of DA has been linked to elevated serum NEFA and BHB concentrations during the first wk after parturition (LeBlanc et al., 2005; Duffield et al., 2009; Chapinal et al., 2011). In the present study, however, plasma NEFA and BHB were not affected by PEG treatment. Indeed, treated primiparous cows tended to have a lower incidence of HYK. Moreover, decreased plasma Ca levels at calving have been associated with a higher risk of developing DA (Massey et al., 1993; Neves et al., 2018). We observed that the proportion of multiparous cows (lactation ≥3) with SCH did not differ between the PEG-treated and control groups. Thus, we cannot explain the detected effect of PEG treatment on DA.

Interestingly, PEG treatment increases the incidence of lameness during the first 30 DIM, although by 60 DIM, there was no significant difference. The differences in the first 30 DIM were seen in multiparous animals with no observed lesion on examination. Milk yield in cows that received PEG and were lame in the first 30 DIM tended to be greater than the lame cows in the CTR group. The observation of lameness in multiparous animals without any observed foot lesions could be associated with side effects in humans. Bone or musculoskeletal pain is the most common adverse effect caused by G-CSF treatment in humans. However, the human dose is significantly higher than in dairy cows (Renwick et al., 2009; Kirshner et al., 2012; Lambertini et al., 2014). The human pegylated molecule, pegfilgrastim, is dosed as a 6 mg dose which would deliver 100 μg/kg in a 60 kg human in contrast to a dose of 20-30 μg/kg administered to dairy cows per label. Additionally, in safety studies investigating administration of the product at 1X, 2X and 3X no adverse events associated with the musculoskeletal system were reported (FDA, 2016; Hassfurther et al., 2015; Canning et al., 2017). Although the exact mechanism of bone pain following G-CSF administration is not fully understood, the following pathophysiological processes have been proposed: (1) quantitative and qualitative expansion of the bone marrow, (2) activation of receptors located in afferent nerve fibers, (3) recruitment and

stimulation of inflammatory cells, and (4) osteoclast activation causing bone resorption (Renwick et al., 2009). More studies are needed to assess the effect of PEG on lameness, and to elucidate the pathophysiological processes involved in this effect.

Although our study has evaluated the effect of PEG administration in a randomized clinical trial with 840 cows, it was limited to a single dairy farm. Therefore, the external generalization of the results must be made cautiously.

CONCLUSIONS

Treatment with PEG did not affect the incidence of mastitis, metritis, puerperal metritis or clinical endometritis, but it increased the incidence of lameness, DA, and combined clinical diseases during the first 30 d postpartum. Administration of PEG increased milk yield in primiparous cows, but did not affect ECM, FCM, and milk composition. Additionally, PEG treatment did not alter reproductive performance. As expected, PEG administration induced postpartum neutrophilia, which also resulted in a higher recruitment of neutrophils to the mucosa of the reproductive tract of cows affected with metritis-PM. These changes in the leukocyte population, induced by PEG administration, were not accompanied by improvements in the health and reproductive performance of treated animals. In fact, the combined disease morbidity was increased for cows treated with PEG.

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CHAPTER 7: Final considerations and future work

The aim of this dissertation was to evaluate the use of recombinant cytokines as a novel therapy to improve health and production in Holstein cows. In chapter 2 we evaluated the effects of a single intrauterine postpartum administration of recombinant bovine interleukin-8 (**rbIL-8**) on uterine health, metabolism, and milk production of Holstein cows. The incidence of postpartum uterine diseases, hyperketonemia (**HYK**), and clinical ketosis were evaluated. We observed that rbIL-8 treatment altered the serum levels of some metabolites, reduced the incidence of HYK, and reduced the incidence of puerperal metritis in multiparous cows. More importantly, intrauterine infusion of rbIL-8 induced a significant long-lasting increase in milk production. In chapter 3, we evaluated the effects of systemic administration of rbIL-8 on glucose clearance and insulin response following a glucose challenge, blood metabolites, immune cell populations, and inflammatory parameters in bull Holstein calves. Glucose tolerance tests were performed 12 h and 7 d after the last rbIL-8 dose. Systemic administration of rbIL-8 induced long-term insulin resistance, elevated rectal temperature, and increased serum concentrations of haptoglobin, metabolites, and white blood cell counts. Findings from Chapter 4 reinforced our findings on increased milk production following rbIL-8-IU treatment. In that study, we evaluated whether recombinant bovine interleukin-8 treatment administered intrauterine (**rbIL8-IU**) or intravenously within 12 h of parturition would increase milk production through effects on insulin resistance, dry matter intake (**DMI**), and/or by altering metabolism. Cows treated with rbIL8-IU produced more milk, fat-corrected milk, and energy-corrected milk yields than controls. Moreover, rbIL8-IU treatment increased DMI. Recombinant bIL-8 treatment did not alter peripheral tissue insulin sensitivity. Additionally, rbIL8-IU reduced the incidence of HYK and diseases combined. Results reported in chapter 4 supports the use of rbIL8-IU treatment shortly after calving to improve health and production responses in lactating cows. Additionally, intravenous administration of rbIL-8 did not suppress DMI or milk production and was not associated with any negative side effects. These results also provide further support to the notion that the observed increase in milk production and DMI as well as improved health following rbIL8-IU are a consequence of a local effect in the uterus.

The most remarkable results from chapters 2 to 4 were the beneficial effects following a single intrauterine infusion of rbIL-8 in Holstein cows on health and production outcomes. More specifically, intrauterine rbIL-8 treatment: (1) reduced the incidence of puerperal metritis, (2) reduced the incidence of postpartum HYK, (3) improved the overall health, (4) increased milk yield in the long term, and (5) increased DMI. Nevertheless, several opportunities for research remain open. First, future studies with a larger sample size and including more than one dairy farm are necessary to confirm whether a reduction on puerperal metritis incidence following intrauterine rbIL-8 treatment is expected. Second, it remains unknown if an early administration of intrauterine rbIL-8, either before parturition or immediately after delivery of the calf, could have a beneficial effect on retained fetal membranes (**RFM**). Thus, studies evaluating an early intrauterine infusion of rbIL-8 on RFM are needed. Third, it will be very interesting to assess the effects of intrauterine rbIL-8 treatment on uterine involution. With that being said, more studies are also needed to evaluate the potential effect of rbIL-8 treatment on resumption of ovarian cyclicity, pregnancy per artificial insemination at first service, and time to pregnancy during lactation. A multi-site randomized clinical trial would be sufficient to test all the above-mentioned hypotheses. Therefore, the study could have the following objectives: (1) to confirm whether a reduction on puerperal metritis incidence following intrauterine rbIL-8 treatment is expected, (2) to evaluate if a single administration of rbIL-8 administered intrauterine right after the delivery of the calf would prevent RFM occurrence, (3) to evaluate if a single administration of rbIL-8 administered intrauterine before parturition would prevent RFM occurrence, decrease the incidence of dystocia, as well as decrease the incidence of stillbirth, and (4) to evaluate the effects of rbIL-8 treatment on uterine involution and reproduction.

Fourth, although we suggested that the reduction on HYK incidence following rbIL-8 treatment might be a result of increased DMI, the exact mechanism by which rbIL-8 treatment improves metabolic health remains unknown. Fifth, the exact mechanism by which rbIL-8 treatment increases DMI remains to be elucidated. Our data demonstrated that a significant increase on DMI and milk yield is observed only following rbIL8-IU. Thus, future studies should

explore the potential mechanism of action underlying the role of rbIL-8 on milk yield, DMI, and postpartum health focusing on a local effect in the uterus exerted by rbIL-8 treatment. Our main hypothesis is that an improved local immune response in the uterus caused by rbIL8-IU might have reduced the endotoxin challenges for these cows and triggered the increased DMI that drove the differences in milk production reported in chapters 2 and 4. A block randomized clinical trial could be used to asses this hypothesis. In this experiment, right after the delivery of the calf Holstein cows would be blocked by parity and randomly assigned to a rbIL-8 treatment group or a placebo control group. Cows in the rbIL-8 group would receive an intrauterine infusion of rbIL-8 and controls would receive saline solution. Blood and uterine lavages would be collected right before treatment, daily for the first 7 days, and weekly until 28 days of parturition. Blood samples would be used to measure lipopolysaccharides (LPS) and LPS binding proteins levels, and uterine lavages would be used to quantify the proportion of neutrophils and to measure LPS concentrations. Collected uterine fluid would be used for aerobic and anaerobic bacterial culture. Moreover, neutrophils from uterine lavages would be harvested and their functionality would be assessed. In addition, blood samples would be used to measure the concentration of TNF-α, a potent anorexigenic inflammatory cytokine highly associated with LPS-induced inflammation.

In chapter 5, we assessed the association between plasma insulin concentration around parturition and milk yield. Blood samples were collected at -7, 0, 3 and 10 d relative to parturition. In addition, colostrum samples were collected. Plasma insulin concentration on d -7 or d 0 was not correlated with colostrum insulin level. Cows were grouped as low insulin (**L-INS**) and high insulin (**H-INS**) based on the median plasma insulin concentration on d 0, 3 and 10. We observed that cows in the L-INS group produced more milk, more fat-corrected milk, and more energy-corrected milk compared with H-INS cows. Results from this chapter highlighted the importance of suppression of postpartum insulin secretion as a key endocrine adaptation to support high milk production.

In chapter 6, we evaluated the effects of treating Holstein cows with pegbovigrastim (**PEG**) on periparturient diseases, milk production, and reproductive performance while exploring the potential mechanism of action. The incidence of periparturient disorders was evaluated within the first 30 DIM. Treating dairy cows with PEG increased the incidence of lameness, displaced abomasum, and combined clinical diseases. Administration of PEG also increased PMN numbers, milk yield in primiparous cows, but did not affect energy-corrected milk. Additionally, PEG treatment did not alter reproductive performance. The results presented in chapter 6 do not support the use of PEG in periparturient Holstein cows to improve health and reproduction. Other studies have shown beneficial effects of PEG treatment on clinical mastitis incidence during the first 30 DIM. Our study was conducted in a single commercial dairy farm with a low incidence of clinical mastitis. Thus, more studies with similar experimental designs than those described in chapter 6 should be conducted to evaluate the effect of PEG on clinical mastitis in farms experiencing a higher risk of clinical mastitis. Bone or musculoskeletal pain is the most common adverse effect caused by granulocyte colony stimulating factor treatment in humans. Interestingly, in our study PEG treatment increases the incidence of lameness during the first 30 DIM. Further studies are needed to assess the effect of PEG on lameness, and to elucidate the pathophysiological processes involved in this effect.